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The Isolation, Characterization, and Evaluation of *Frankia* strains

P. Normand, M. Lalonde, J.A. Fortin, and L. Chatarpaul

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PETAWAWA NATIONAL FORESTRY INSTITUTE

The Petawawa National Forestry Institute (PNFI) was formed on April 1, 1979, as the result of an amalgamation of the Petawawa Forest Experiment Station with the Ottawa-based Forest Management and Forest Fire Research Institutes. The Forestry Statistics and Systems Branch was established at PNFI in 1980.

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Through the FORSTATS program, which involves all regional establishments of the Canadian Forestry Service, the FSSB coordinates the acquisition and publication within the CFS of national statistics on the forest of Canada.

Every five years, the FSSB publishes Canada's Forest Inventory; the official report on the location, extent, species, and condition of the forest resource. In addition, the FSSB is working closely with the provinces to expand the information available on changes to the forest from fire, harvesting, insects and disease, and from forest management activities. This information is essential to the development of sound policies for the improved management of this important and renewable natural resource.

COVER

A nodule of *Alnus* from which *Frankia* has been isolated in pure culture.

THE ISOLATION, CHARACTERIZATION, AND EVALUATION OF *FRANKIA* STRAINS

Information Report PI-X-30

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Abstract

Osmium tetroxide (OsO_4) was used as the sterilizing agent to isolate the nitrogen-fixing endophyte *Frankia* from nodules of host plants. This treatment resulted in a pure culture collection of 250 *Frankia* isolates from several specis of *Alnus* (*crispa, rugosa, viridis, glutinosa,* and *serrulata*) and from other actinorhizal species from Quebec (*Myrica gale* and *Shepherdia canadensis*). *Frankia* isolates from *A. viridis* and *S. canadensis* are reported here for the first time.

Sugars from whole-cell hydrolysates of the organisms were analysed by gas liquid chromatography (GLC) to identify strains previously screened on the basis of morphology and infectivity. This technique proved especially useful for those isolates whose morphology was atypical (ACN8, ARgN22, SCN9, and SCN10). It was demonstrated that sugar analysis (2-0-methy-D-mannose, in particular) can be important in resolving the taxonomy of *Frankia*.

The nitrogen-fixing efficiency of some *Frankia* strains was evaluated by inoculating *A. crispa*. It was found that the efficiency of organisms was not influenced by the host from which they were first isolated. However, significant differences between some isolates of the same provenance occurred. It was also found that those strains of *Frankia* which do not sporulate were more effective in fixing nitrogen than those which do. It is proposed that the terms "Type P" and "Type N" be used to designate spore positive (Sp^+) and spore negative (Sp^-) *Frankia* strains, respectively. Further, it is recommended that "sporulation type" be used as a valid basis for species definition in the genus *Frankia*.

Résumé

Le tétroxyde d'osmium (OsO_4) a servi de stérilisant pour l'isolement de *Frankia*, endophyte fixateur de l'azote dans les nodules de plantes hôtes. On a ainsi obtenu 250 isolats en culture pure à partir de plusieurs *Alnus* (*A. crispa, A. rugosa, A. viridis, A. glutinosa* et *A. serrulata*) ainsi que d'autres espèces non-légumineuses du Québec capables de fixer l'azote (*Myrica gale* et *Shepherdia canadensis*). C'est la première fois que de tels isolats sont signalés chez *A. viridis* et *S. canadensis*.

Les sucres de l'hydrolysat de cellules entières des organismes ont été analysés par chromatographie en phase gazeuse afin d'identifier de façon positive les souches préalablement sélectionnées d'après leur morphologie et leur infectivité. Cette technique s'est révélée particulièrement utile dans le cas des isolats dont la morphologie était atypique (ACN8, ARgN22, SCN9 et SCN10). De plus l'analyse des sucres (du 2-0-méthyle-D-mannose notamment) peut grandement aider à déterminer la taxonomie de *Frankia*.

La capacité à fixer l'azote de certaines souches de *Frankia* a été mesurée après leur inoculation sur *A. crispa*. On a constaté qu'elle ne dépendait pas de l'hôte dont les organismes avaient d'abord été isolés. Toutefois, certains isolats de même origine différaient grandement sur ce point, et les souches de *Frankia* qui ne sporulaient pas fixaient mieux l'azote que les autres. Il est proposé de désigner par "type P" et "type N" les souches de *Frankia* à sporulation positive (Sp^+) et négative (Sp^-) respectivement, et il est recommandé que le type de sporulation serve à la définition taxonomique du genre *Frankia*.

THE ISOLATION, CHARACTERIZATION, AND EVALUATION OF *FRANKIA* STRAINS

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1 INTRODUCTION

In recent years, pressures on the forest resources of the world have increased greatly. Not only are the forests providing more conventional forest products, but a world-wide energy crisis has directed attention on forests as a source of liquid, solid, and gaseous fuels to replace fossil fuels, at least in part. More efficient, intensive silvicultural and logging systems are being used to produce more fibre per unit land area on sites with reasonable access. Increasing yield by utilizing the whole tree, and shortening rotations, will inevitably create greater nutritional stresses on forest soils which are generally low in fertility. These soils must therefore be managed if productivity is to be optimal.

Nitrogen is essential to life. Indeed, this element is limiting to productivity in many ecosystems (Campbell 1977). Nitrogen occurs in amino acids, nucleic acids, chlorophyll, cytochromes, vitamins, and other molecules in living tissue.

In a mature community, elements such as phosphorus or sulfur may become limiting (Postgate 1979) but in disturbed areas such as glacial tills (Ugolini 1968), sand dunes (Dommergues 1966), or burnt-over sites (Wollum and Davey 1975) soils are often deficient in nitrogen. It is generally accepted that most forests in Canada will respond favourably to nitrogen fertilization.

In recently clear-cut forests nitrogen is often lost in amounts that vary according to harvest method and rotation period (Tran Van Nao 1974). The removal of merchantable stems represent a net loss of between 40 to 400 kg N ha^{-1}. The loss of N is significantly higher when the whole tree is harvested and when rotation period is reduced from 40 to 20 years (Tran Van Nao 1974). Armson (1977) noted that loss of nitrogen from forest ecosystems is generally of greater significance than loss of phosphorus or potassium.

Biological nitrogen fixation is being considered as a means of increasing input of nitrogen in intensively managed forest systems. Actinomycete-nodulating nitrogen-fixing symbioses, such as *Alnus/Frankia*, are good candidates for forestry applications (Tarrant and Trappe 1971).

Several governments as well as private companies (Crown Zellerbach, Weyerhauser, and McMillan Bloedel) are funding research in that field (Rottink 1980). Mixed cultures, crop rotations, and utilization of nitrogen-fixing trees and shrubs in disturbed areas are among the many ways envisioned to transfer nitrogen fixed by a symbiotic species to the ecosystem, and for producing biomass as an energy source (Rottink et al. 1979). *Alnus crispa* and *A. rugosa* are currently under consideration for energy plantations in Quebec (Fortin et al. 1979).

Relatively little is known about the biology of *Frankia*, the endophyte which forms a nitrogen-fixing symbiosis with *Alnus* spp. This is partly due to past difficulty encountered in the isolation of *Frankia* in pure cultures. Hall et al. (1979) suggested that a program of isolation, characterization, and evaluation of *Frankia* is a prerequisite to the use of this symbiosis in forestry. For example, any serious work on the selection of superior strains of *Frankia* is dependent upon access to a culture bank of strains from the location where application is considered.

The aim of this study was to establish a major collection of *Frankia* strains in pure culture by adding to collections of Lalonde et al. (1981) new isolates from host plants native to eastern Canada. Secondary objectives were (i) to confirm the identity of isolated strains by using a biochemical assay, (ii) to evaluate the nitrogen-fixing efficiency of some of the strains by reinoculating *Alnus crispa* and, (iii) to evaluate the efficiency of surface sterilizing agents. A review of pertinent literature is also presented.

2 LITERATURE REVIEW

2.1 The nitrogen cycle

The nitrogen cycle is one of the most complex of the biogeochemical cycles (Campbell 1977). Nitrogen exists in many different compounds at different oxidation levels and is transformed in many ways, mostly by biological means (Postgate 1978). Gaseous nitrogen (N_2) makes up almost 80% of the atmosphere but is in a form unavailable to most organisms including plants. Available nitrogen, usually in the form of ammonium and nitrate ions, are lost from the ecosystem by processes such as denitrification, volatilization, leaching, or through biomass removal (Russel 1973). Under steady state conditions, these losses can be compensated by chemical and biological fixation and by precipitation inputs of nitrogen oxides and ammonium (Wollum and Davey 1975).

Present trends in forestry toward intensified management in order to produce more fibre may create chronic nitrogen deficiencies in soil. Natural inputs through mineralization and precipitation would not be sufficient to fulfill the forest's nitrogen demands. Two possibilities remain: (i) to supply nitrogen in the form of chemical fertilizers or, (ii) to employ new forestry practices such as biological nitrogen fixation as suggested by Beuter (1979). Under certain conditions the use of chemical fertilizers may have some advantages. However, it is unlikely that current cost and demand for fertilizers will allow large scale fertilization of Canada's massive forests. The use of biological techniques such as nitrogen-fixation to maintain fertility shows good potential (Rottink et al. 1979), especially in intensively managed plantations or short rotation forestry.

2.2 Nitrogen fixation

The earth, like Mars or Venus, probably acquired its atmospheric and soil nitrogen through slow degradation of the crust (Pollack 1981) but this input is now marginal. The same is true for input through rainfall which accounts for only 1 to 15% of the nitrogen requirement (Armson 1977). Fixation, therefore, constitutes on a global basis the most important nitrogen input.

Nitrogen fixation is accomplished by two main pathways: (i) the chemical Haber-Bosch process or, (ii) the enzymatic pathway involving nitrogenase, an enzyme found only in some prokaryotes (Ruvkun and Ausubel 1980). At present, it is estimated that about 100 billion metric tons of nitrogen are fixed annually, half of it in factories by the costly Haber-Bosch process that consumes methane to reach the 500°C and 200-1000 atmospheres required to complete the reaction. On the other hand, many nitrogen-fixing microorganisms fix 50 billion metric tons at normal pressure and temperature (Campbell 1977).

Two main groups of plants have established a symbiotic association with nitrogen-fixing prokaryotes (Maynard 1980). These are the legumes with *Rhizobium*, and the actinorhizal plants with *Frankia*. Other nitrogen-fixing symbioses exist, such as that of the cycads or the foliar nodules of *Psychotria* (Van Hove 1976), but these symbiotic systems are not widespread, are limited to the tropics, and have low potential for commercial exploitation in the near future. The boreal forest contains only herbaceous legumes (Marie-Victorin 1964) and few of these find practical applications such as underplanting (Zavitkovski et al. 1979) because, among other reasons, they are less frost-hardy than alder species (Maynard 1980). On the other hand, actinorhizal species are pioneer plants that thrive on a variety of extreme ecosystems as summarized in Table 1.

2.3 Isolation of *Frankia* strains

Since the turn of the century, more than 40 papers have been published on unsuccessful attempts at isolating *Frankia* (Baker and Torrey 1979). Some of the reasons cited for these isolation failures are: i) a slow growth rate of actinomycetes in general and of *Frankia* in particular relative to eubacteria (Baker and Torrey 1979); ii) the presence in the nodules of significant amounts of phenolic compounds that are possibly inhibitory to *Frankia*. These compounds are released in the growth medium upon crushing (Baker et al. 1979a); iii) the need for a massive inoculation of the medium (Quispel and Burggraaf 1981); iv) the uncertainty concerning *Frankia*'s oxygen demands (Lalonde et al. 1975); v) requirements for growth that were not known due to confusion concerning the identity of the endophyte (Baker and Torrey 1979); vi) special growth requirements of certain strains (Quispel and Burggraaf 1981), especially when cultured outside the nodule (Lalonde and Calvert 1979); and finally, vii) according to Baker and Torrey (1979) a general underestimation of the complexity of isolating *Frankia* in pure culture.

To illustrate the difficulties encountered in isolating *Frankia*, the following comparison is made to the culture of *Rhizobium* on a few key parameters:

(i) *Growth rate: Rhizobium*, like many eubacteria, produces visible colonies on agar after 2-3 days, whereas *Frankia* takes anywhere between 2 weeks and 2 months. While some actinomycetes such as species of *Streptomyces* grow rapidly on agar, others are much slower (Athalye et al. 1981). *Frankia*, therefore, will be overgrown by almost any contaminant. The fact that *Frankia* grow slower on agar than in liquid medium further complicates isolation (Callaham et al. 1978).

(ii) *Phenols:* Actinorhizal plants are all woody dicots, except *Datisca* (Akkermans and Houwers 1979), and the biochemistry of actinorhizal nodules is different from that of legume nodules; the amount of phenolic compounds is more important in the former than the latter. This is evident by the reddening of nodules upon crushing. Use of polyvinyl pyrrolidone (Loomis and Battaille 1966), or techniques such as Sephadex chromatography (Baker et al. 1979a), or sucrose-density gradient centrifugation (Baker et al. 1979b) is necessary to prevent oxidation of phenols which appear to have an inhibitory effect on the growth of *Frankia*.

(iii) *Massive inoculation:* A massive inoculation of medium is not necessary to isolate *Rhizobium* strains in pure culture. However, a requirement for massive inoculation with *Frankia* has been noted by Quispel and Burggraaf (1981) and, because of the slow growth rate of *Frankia* relative to contaminants, thorough sterilization of nodule surface is necessary.

(iv) pO_2: Before the first isolation was done by Callaham et al. (1978), it was thought that *Frankia* was microaerophilic (Lalonde et al. 1975) because of the large number of layers surrounding the endophyte (Lalonde and Knowles 1975a), and because of the lack of haemoglobin (Bond 1951) to protect the O_2-sensitive nitrogenase. Many isolation attempts may have failed for that reason (Baker and Torrey 1979). It is now known that the pO_2 of the nodule intercellular spaces is about 20% (Tjepkema 1979) and that actinorhizal nodules also have an optimal pO_2 for fixation of about 20% (Sloger 1968, Fessenden et al. 1973). Tjepkema et al. (1980) noted that *Frankia* appears to be microaerophilic for the *in vitro* nitrogenase expression. Other reports have

Table 1. Some common habitats of actinorhizal species

Habitat	Species	Source
Glacial tills	*Dryas drummondii*	Crocker and Major (1955)
	Shepherdia canadensis	Crocker and Major (1955)
	Alnus spp.	Crocker and Major (1955)
Lava deposits	*Myrica faya*	Smathers and Gardner (1979)
	Alnus spp.	Hunt and Trappe (1981)
	Coriaria spp.	Silvester et al. (1979)
Clear-cut areas	*Alnus* spp.	Resch (1980)
Burnt-over areas	*Ceanothus*	Youngberg and Wollum (1976)
	Cercocarpus	Hoeppel and Wollum (1971)
High altitudes	*Alnus viridis*	Moiroud and Copellano (1979)
Deserts	*Chamaebatia foliolosa*	Heisey et al. (1980)
	Cowania mexicana	Righetti and Munns (1980)
	Datisca glomerata	Winship and Chandhary (1979)
	Purshia spp.	Dalton and Zobel (1977)
Sand dunes	*Hippophaë rhamnoides*	Akkermans (1971)
	Casuarina spp.	Dommergues (1966)
	Myrica spp.	Morris et al. (1974)
Peat deposits	*Myrica gale*	Bond (1951)
Mine spoils	*Alnus glutinosa*	Carpenter and Hensley (1979)
Gravel deposits	*Alnus viridis*	Benecke (1970)
	Dryas drummondii	Petrides (1958)
Chalk cliffs	*Elaeagnus commutata*	Marie-Victorin (1964)

described nitrogenase as anaerobic (Benson et al. 1979) or aerobic for *in vitro* vegetative growth (Burggraaf et al. 1981). However, Baker et al., (1981a) and Lechevalier and Lechevalier (1979) prefer to classify *Frankia* as microaerophilic to aerobic.

(v) *Identity of the endophyte:* It has been known for a long time now that *Rhizobium* is an eubacteria and its simple nutritional requirements are well defined (Vincent 1977). *Frankia*, on the other hand, has been diversely classified as a mycorrhizal fungus, a slime mold, an eubacteria, or an actinomycete, and thus growth conditions used for pure culture isolation were not optimal (Baker and Torrey 1979). Investigations into the biochemistry of *Frankia* has only recently been initiated (Blom 1981).

(vi) *Nutritional requirements:* For all *Rhizobium* species, a medium with yeast extract and a sugar is sufficient (Vincent 1977) but *Frankia* isolates from *Alnus*, for example, have shown a large diversity in growth requirements (Quispel and Burggraaf 1981). For instance, an isolate designated as Avcl1 has shown a high versatility in carbon and nitrogen sources (Blom 1981). This was expected because it had been isolated on "*Frankia* broth" (Baker and Torrey 1980), a simple medium (Table 2). At the other end of the spectrum, strains isolated from *A. glutinosa* Sp^+ and Sp^- nodules have shown negligible to slow growth unless supplemented with an alcoholic lipid-extract from alder roots (Quispel and Burggraaf 1981). This is similar to the improved growth of some Sp^+ isolates observed when subcultured in FBS (Fetal bovine serum, Gibco, Grand Island, NY) – supplemented Qmod B medium (Lalonde unpubl.).

Usually the first growth out of the nodule is slower than that occurring upon subsequent culturing. Growth requirements are also more critical during this transition phase to new artificial conditions (Lalonde unpubl.).

Some very complicated techniques to isolate *Frankia* in pure culture have been reported (Quispel 1955, 1979, Callaham et al. 1979, Burggraaf et al. 1981) and simpler methods such as that of Gel'tser (1965) have been claimed. However, none of these has proven effective. Table 3 compares five more successful isolation methods.

It is generally agreed that the first successful isolation was that of Callaham et al. (1978). They macerated *Comptonia peregrina* nodules in a pectinase-cellulase solution to liberate hyphae and vesicles clusters. This operation yielded *Frankia* colonies many weeks later. Since then, other methods that have resulted in successful pure culture isolation of *Frankia* strains have been developed. For example, Lalonde (1979a), using a serial dilution method, isolated two *Frankia* strains, $ACNI^{AG}$ and $CPNI^{AG}$, from *A. glutinosa* seedlings using Qmod B medium (Lalonde and Calvert 1979). The same method was used by Gauthier et al. (1981) with *Casuarina* nodules.

Baker et al. (1979b) described a method based on a sucrose-density gradient centrifugation to separate hyphae and vesicles from nodule debris, phenols, and other contaminants and succeeded in isolating Eul1 strains from *Elaeagnus umbellata* and Avcl1 strains from *Alnus crispa* (Baker et al. 1980) using "*Frankia* broth" (Baker and Torrey 1979). The same method was used by Lechevalier (pers. comm.) to isolate Mpl1 and Airl from *Myrica pensylvanica* and *A. rugosa* respectively.

Burggraaf et al. (1981) have developed a selective incubation procedure to isolate *Frankia* strains from *A. glutinosa* and *M. gale*. Briefly, their method involves sterilizing the surface of nodules with 0.1% bromine, dissecting the nodules to remove the tannin-rich epidermis, and incubating to ensure that axenic conditions are met. The nodules were crushed, filtered, and the contents incubated in top-layer agar (0.8%) containing peptones and lipids over a bottom agar (1%) that contained the other ingredients of the Qmod medium.

Lalonde et al. (1981) described an effective method using osmium tetroxide (OsO_4) to kill all surface contaminants as well as those in epidermal fissures that often escape when a conventional sterilization method is used. A 3% W/V aqueous solution of OsO_4 is applied to the nodules which are then crushed in PVP to absorb phenols. Thereafter, Qmod B tubes are inoculated with the slurry. Using this method *Frankia* strains from *Alnus glutinosa, A. serrulata, A. tenuifolia, A. rubra, Elaeagnus umbellata*, and *E. augustifolia* were isolated (Lalonde et al. 1981).

Many laboratories have been, and still are, isolating *Frankia* strains from the genera *Alnus, Myrica, Comptonia, Elaeagnus, Shepherdia, Hippophaë, Purshia, Ceanothus*, and *Casuarina* (See Table 4).

Collections of isolates of *Frankia* are important for biological research and practical applications. These could include biomass production, recolonization of mine spoils and other disturbed sites, as well as in mixed plantations and in soil amelioration efforts. A partial list of collections of *Frankia* given in Table 4 may not be complete because not all results have been published.

Table 2. Composition of media used to culture *Frankia*

	"A" 1st isol.	"B" *Frankia* broth	"C" Qmod B	"D" TOT	"E" Kalininskaya
Yeast extract	500	5000	500	–	80
FeNaEDTA	–	–	–	10	–
$FeCl_3.6H_2O$	–	–	–	–	10
ferric citrate	–	–	0.01	–	–
NaH_2PO_4	–	–	200	–	–
$MgSO_4.7H_2O$	200	–	200	100	290
KH_2PO_4	–	–	–	1000	870
K_2HPO_4	500	–	300	–	1670
$CaCO_3$	500	–	100	–	–
$CaCl_2$	–	–	–	10	70
NaCl	100	–	–	–	480
KCl	–	–	200	–	–
malate	–	–	–	–	2320
succinic acid	–	–	–	1200	–
mannitol	1000	–	–	–	–
sucrose	20000	–	–	–	–
dextrose	–	10000	–	–	–
glucose	–	–	10000	–	–
casamino acids	–	5000	–	–	–
bactopeptone	–	–	5000	–	–
edamin	1000	–	–	–	–
lecithin	–	–	5	–	–
vitamin B_{12}	–	1.6	–	–	–
pyridoxine	0.1	–	–	0.5	–
thiamine	0.1	–	–	0.1	–
nicotinic acid	0.5	–	–	0.5	–
biotin	–	–	–	–	0.02
$MnSO_4.(xH_2O)$	25(1)	4.5(1)	0.8(7)	–	3(4)
$MgCl_2.2H_2O$	–	–	–	1.8	–
H_3BO_3	100	1.5	1.5	2.9	5
$ZnSO_4.(xH_2O)$	10(1)	1.5(7)	–0.6(7)	0.2(7)	0.2(7)
$Na_2Mo_4.2H_2O$	0.25	0.25	–	0.025	5
$(NH_4)_6Mo_7O_{24}.4H_2O$	–	–	0.2	–	–
$CuSO_4.(xH_2O)$	0.025(1)	0.04(1)	0.1(7)	–	–
$CoSO_4.7H_2O$	–	–	0.01	–	0.002
pH	7	6.4	6.8-7.0	6.8	7.0

Table 2 presents the media used by Callaham et al. (1978): Medium "A", Baker and Torrey (1979); medium "B", Lalonde and Calvert (1979); medium "C", Tjepkema et al. (1980); medium "D" and by Gauthier et al. (1981); medium "E". All quantities are expressed in mg L^{-1}. Media "A", "B", and "C" have been used for isolation and medium "D" for *in vitro* nitrogenase expression.

Table 3. Comparison of the five isolations methods

	Enzymatic digestion	Serial dilution	Sucrose gradient	Selective incubation	OsO_4 treatment
Complexity[a]	very high	low	high	high	low
Success rate[b]	very high	low	medium	medium	high
Growth rate[c]	low (2 mo.)	low (2 mo.)	low (2 mo.)	low (2 mo.)	high (2 wks.)
Number of distinct isolates from nodule	low	low	low	high	high
Toxicity	low	low	low	medium	high

a) time, manipulations, and material required.
b) based on Baker et al. (1979b) for sucrose gradient, on Quispel (1979) and Quispel and Burggraaf (1981) for selective incubation, on Lalonde (1979a) and our laboratory for serial dilution, on Lalonde et al., (1981) and our laboratory for the Oso_4 treatment. Does not apply for enzymatic digestion (Callaham et al. 1978, Baker and Torrey 1979), that has succeeded only once.
c) time necessary for the appearance of colonies visible to the naked eye.

2.4 Characterization of *Frankia* isolates

The first isolate obtained was characterized morphologically and by its capacity to form nodules; in short, by fulfilling Koch's postulates (Callaham et al. 1978). Morphology is still the most widely-used criterion.

2.4.1 Media and morphology

The different media used to grow *Frankia* have an effect on morphology and rate of growth. For instance, according to Blom et al. (1980), a modification of the lipid source—lecithin replaced by Tween (Sigma Chem. Co., St. Louis, Mo.) in Qmod b—will double the total organic carbon, and according to Lalonde and Calvert (1979), fetal bovine serum will inhibit sporulation. On the other hand, *Frankia* is a pleomorphic microorganism whose development is sequential (Lalonde and Calvert 1979). Changes in growth conditions will greatly influence its morphology. For example, upon isolation, the number and size of sporangia increase and the number of vesicles decreases relative to the endophytic stage (Callaham et al. 1978). Moreover, some *Frankia* strain are more sensitive than others to the presence of some lipid compounds in the medium (Quispel and Burggraaf 1981). It appears then that the most complete medium should be chosen, especially for isolation, in order to avoid selecting for simpler growth requirements. The medium chosen by Lalonde and Calvert (1979) (Table 2, "B"), and by Quispel and Burggraaf (1981), appear to be suitable for a large spectrum of strains. This medium permits the isolation of diverse strains, including Sp^+ strains, with a high rate of success. And because it appears that modifications in growth conditions cause morphological changes for many actinomycetes in subculture (Waksman 1959), the medium used for isolation should be used for subsequent subculturing unless modifications in either morphology or metabolism are required. (Tjepkema et al. 1980).

Having chosen a precise medium for isolation and subculturing, one can use morphology to identify an isolate as *Frankia* if most morphological criteria described by Lalonde and Calvert (1979) are present; for example, branched septate hyphae, pedunculate vesicles, and sporangia containing non-motile spores.

2.4.2 Infectivity and biochemical tests

Another criterion used to identify an isolate as *Frankia* is the ability to induce the formation of nitrogen-fixing nodules on a compatible host-plant (Lalonde 1979a, Lechevalier and Lechevalier 1979).

Biochemical criteria have also recently been adapted to identify *Frankia*. These include the Ouchterlony produre that permits inclusion of a given isolate into a serogroup (Baker et al. 1981b), sugar chromatography of whole-cell hydrolysates that yields

Table 4. List of *Frankia* collections

Location	Collector/s	Source of *Frankia* isolates	
Laval	Lalonde and Normand	*Alnus crispa*	*Myrica gale*
		rugosa	*Comptonia peregrina*[H]
		tenuifolia	*Elaeagnus umbellata*
		rubra	" *angustifolia*
		viridis	*Shepherdia canadensis*
		serrulata	
		glutinosa	
Middlebury	Baker	*Alnus rugosa*	*Myrica gale*
		rubra	" *cerifera*
		rugosa	*Elaeagnus commutata*
		sinuata	" *umbellata*
		Comptonia peregrina	*Purshia tridentata*
Netherlands	Quispel and Burggraff	*Alnus glutinosa*	*Myrica gale*
		crispa[M]	*Comptonia peregrina*[H]
Rutgers	Lechevalier Others	*Alnus rugosa*	*Ceanothus americanus*
		Myrica pensylvanica	
Harvard Forest	Berry, Callaham, and Torrey	*Alnus rubra*	*Comptonia peregrina*
Weyerhauser	Carpentier and Robertson	*Alnus rubra*	*Alnus sinuata*
Dakar	Gauthier, Domergues, and Diem	*Casuarina equisetifolia*	*Hippophaë rhamnoides*
Lyon	Moiroud and Simonet	*Alnus viridis*	*Alnus rugosa*[L]
		incana	" *rubra*[L]
		glutinosa	" *crispa*[L]
Connecticut	Benson	*Alnus rugosa*	

Letter placed at the upper right indicates the provenance of the strains. (H = Harvard; M = Middlebury; L = Laval).

a "fingerprint" which can be compared to that of a confirmed *Frankia* (Mort and Lalonde 1981), and the establishment of the cell wall structure. In *Frankia*, the cell wall structure is of type III because it comprises mesodiaminopimelic acid (meso-DAP), alanine, glucosamine, an unknown amino acid, and an unknown hexose (Lechevalier and Lechevalier 1979) which is probably 2-0-methyl-D-mannose (Mort and Lalonde 1981).

Baker et al. (1981a) listed 10 criteria that should help define given isolates as *Frankia*. These are; 1) slow growth (up to 8 weeks for the appearance of visible colonies), 2) microaerophilic to aerobic, 3) septate hyphae, 4) vegetative sporangia and non-motile spores,

5) sometimes pigmented, 6) fixes nitrogen through specialized vesicles, 7) Gram-positive, 8) have a multilamellar envelope exterior to the cell wall, 9) contain meso-DAP acid and, 10) have a cross-reaction with anti-*Frankia* serum.

2.4.3 Characterization of isolates using sugar analysis

Isolates cannot be easily characterized, even though guidelines have been proposed (Baker et al. 1981a). The most practical criteria are morphology and infectivity on a compatible host-plant (Lechevalier and Lechevalier 1979) but it was felt that this method alone was inadequate for differentiating closely-related strains. Other techniques such as ability to sporulate should be used. This will be discussed later in this paper.

Sugars have various functions in cells; among others things, they act as energy reserves and enter structures such as cell walls (Brock 1974). Some of these sugars, like glucose, are quite common in many microorganisms and therefore not very useful in taxonomy. On the other hand, there exist sugars such as madurose (3-O-methyl-D-galactose) which is only found in some rare actinomycetic genera like *Actinomadura* (Lechevalier and Gerber 1970) and is therefore very useful in classification and identification of strains. The bacterial cell wall is made up of, besides sugars, hexosamines and amino acids (Brock 1974). The identity of these different constituents is used to define cell wall types especially in actinomycetes. *Frankia* strains, as mentioned before, have type III cell walls. These criteria appear to be valid in general but not very practical when hundreds of isolates have to be defined. However, the presence of a sugar unique to all *Frankia* isolates could be used, in conjunction with the infectivity test and the presence of the morphological features mentioned before, to properly characterize an isolate as *Frankia*.

Carbohydrates or sugars form a complex family of diverse organic compounds, from the four-carbon tetrose to polymers composed of hundreds of thousands of units (Morrison and Boyd 1973). Because they are present in all microorganisms (Brock 1974), sugars could be used in their taxonomy (Lechevalier and Lechevalier 1970) in much the same way as DNA (Okanishi and Okami 1976), proteins (Kersters 1970), or secondary metabolites such as antibiotics or terpenes (Turner 1973).

Mort and Lalonde (1981) found a sugar in whole cell hydroloysates of *Frankia* that has not been previously reported for any microorganism. This sugar has been identified by gas-liquid chromatography of alditol acetates and trimethylsilyl derivatives, and by mass spectrometry, as 2-O-methyl-D-mannose. It has been found in all *Frankia* strains tested and could therefore be used as a marker sugar. There also exists a sugar spectrum characteristic of the genus *Frankia*—strains that are very different morphologically. ACNI^{AG} and EANIpec for instance, both have the following eight sugars: rhamnose, ribose, xylose, 2-O-methyl-D-mannose, mannose, galactose, glucose and inositol (Lalonde and Mort pers. comm.). In the present study it was decided to use the sugar spectrum to confirm identification of *Frankia* isolates with atypical morphological characteristics such as ACN8, ARgN22, SCN9a, and SCN10a. It also became necessary to determine whether 2-O-methyl-D-mannose was associated with the cell wall and to check its absence in other actinomycete genera, hence confirming this as an identification tool for *Frankia* isolates.

2.5 Evaluation of *Frankia* isolates

Evaluation of isolates could include determination of the ability to metabolize sugars or amino acids, resistance to heavy metals, or defining optimum conditions for N-fixation and growth. Because the most immediate purpose of the isolation program is improvement of the symbiosis in fixing nitrogen, evaluation here specifically refers to the ability of isolates to fix nitrogen and allow growth of the host plant. Such an evaluation will have direct relevance in determining which strain should be used in large-scale inoculation for afforestation of *Alnus crispa* and *A. rugosa* or other species on disturbed areas. Nitrogen-fixing systems are commonly evaluated using the acetylene reduction test, $^{15}N_2$ assays or a bioassay (biomass production under N-free conditions).

2.5.1 Acetylene reduction test

Van Dijk (1978) attempted to differentiate between Sp+ and Sp− strains using the acetylene reduction assay but did not find a statistically valid difference. It must be pointed out that the acetylene reduction test is a short-term measure of a parameter that fluctuates throughout the day (Wheeler 1969), and throughout the year (Pizelle and Thierry 1977) depending upon temperature (Waughman 1972), and pO_2 (Fessenden et al. 1973). Consequently, variance is high even among clones (Dawson and Gordon 1979).

2.5.2 $^{15}N_2$ assays

The measure of fixation of $^{15}N_2$ is more complex. It also has the disadvantage of being time dependent and expensive (Bergersen 1980).

2.5.3 Biomass production under N-free conditions

Recently, Dawson and Sun (1981), and McLean (1981) used the acetylene reduction assay and different parameters of growth under N-free conditions to evaluate the efficiency of nitrogen fixation by actinorhizal plants. Dawson and Sun (1981) found that a biomass assay (height and diameter increments) of seedlings grown in total absence of fixed nitrogen was better than just nodule weight and number in testing the efficiencies of some *Frankia* strains in symbiosis with three *Alnus* species. Their results with the acetylene assay were still less satisfactory. McLean (1981), working with *Shepherdia canadensis*, found that measurement of root stem and total nitrogen were the best indicators of nodulation and of nitrogen status of the plants.

Maynard (1980) used four provenances of *Frankia* in the form of crushed nodules to inoculate *Alnus glutinosa* seedlings. After a few months of growth under conditions of total absence of fixed nitrogen, the plants were evaluated using acetylene reduction and total biomass assays. The total biomass assay enabled Maynard to demonstrate significant differences in efficiency due to the strains being Sp^+ or Sp^-. When the acetylene as assay was used these differences were not significant at the 0.05 level.

2.6 Sterilizing agents for axenic germination of *Alnus crispa*

Due to its ability to fix nitrogen in symbiosis with *Frankia*, alders (*Alnus* spp.) are presently considered for biomass production as intermediates in crop rotations, and as nurse trees (Tarrant and Trappe 1971). Studies on actinorhizae currently require the use of axenic host plant seedlings. Recently Knowlton et al. (1979) hypothesized that some bacteria "helped" the infection process of roots by *Frankia* sp., causing curling and folding of root hairs at the point of penetration by the endophyte. To test this hypothesis it is first necessary to find the best surface sterilization procedure. The presence of fungi and bacteria has also been found to adversely affect early growth of *Alnus crispa* (Ait.) Pursh. seedlings (pers. obs.) used in effectivity (Lalonde and Calvert 1979) and infectivity tests (Lalonde 1979a).

Different methods have been used for surface sterilization of alder seeds. On *A. glutinosa*, Becking et al. (1974), Rodriguez-Barrueco (1968), Skeffington and Stewart (1976), and Lalonde (1979c) used sodium or calcium hypochlorite at concentrations varying from 2 to 5.25% for 10 to 45 min. Again on *A. glutinosa*, Quispel (1954), Angulo Carmona (1974), Van Dijk and Merkus (1976), as well as Lalonde and Quispel (1977) used 0.1% bromine for periods of 5 to 15 min. On *A. crispa* (Ait.) Pursh, Lalonde and Fortin (1972) used 5.25% sodium hypochlorite for 45 min and, more recently, Jobidon and Thibault (1980) suggested 10 to 15 min treatments with 1% merthiolate. Finally Neal et al., (1967) recommended treating *A. rubra* seeds with 30% H_2O_2 for 30 sec to 30 min. However, none of the authors cited above have justified the use of their procedures over others. In other words no one has so far compared the efficacy of the different sterilizing agents.

Erratic results were often obtained by using sodium hypochlorite (NaClO) in our laboratory. In some cases most of the seeds germinated but were contaminated. With longer treatment periods, surface sterilization was achieved but seeds did not germinate or seedlings were malformed. Consequently, a thorough comparison was made of different surface sterilizing agents applied for different periods of time on the seeds of *A. crispa*.

3 MATERIALS AND METHODS

3.1 Isolation of strains

Nodules from which isolation was performed were obtained as follows:

1. Greenhouse grown plants, from surface sterilized seeds, planted in Turface (International Minerals and Chemicals Corp., Des Plaines, Ill.), fertilized with N-free Crone solution (Lalonde and Calvert 1979) and inoculated with soils from the field.
2. Uprooted plants from the field, transplanted in pots containing Turface and kept in the greenhouse.
3. Directly from nodule clusters excised *in situ* and treated within a few hours.

3.1.1 Surface sterilization

Nodule lobes were washed with water, dried with blotting paper, and immersed in a 3% W/V aqueous OsO_4 solution for periods ranging from 5 sec to 6 min (Lalonde et al. 1981). After three successive rinsings in sterile distilled water and 5-15 minutes incubation in sterile PVP-PBS solutions at 0.1% PVP concentration for *A. crispa, A. viridis, M. gale, S. canadensis* and 3% W/V for *A. rugosa, A. glutinosa* and *A. serrulata*, the single lobes were crushed in new sterile PVP-PBS solutions and transferred to 20 × 150 mm tubes containing 14 mL of Qmod B medium (Lalonde and Calvert 1979) which incorporated 5 mg L^{-1} of L-α-lecithin. A total of 50 tubes was normally used for each provenance of nodules and these were incubated at 27° C.

Upon emergence of fluffy whitish outgrowths on the blackened surface of nodules, naked eye observation of the tubes and a microscopic observation using the 100X objective of the phase or interference contrast optics enabled presumptive identification of the isolates as *Frankia* (Lalonde et al. 1981).

3.1.2 Subculture of isolates

Ten tubes containing pure presumptive *Frankia* isolates were chosen at random, sealed with a rubber serum cap, and the colonies broken with a syringe and needle (20 G). For the first subculture, four fresh Qmod B tubes were inoculated. The dilution factor, low at first, could be increased up to 1 to 50 or more upon the second subculturing in the case of Sp^- isolates. For some Sp^+ from *Alnus rugosa, A. glutinosa*, and *A. serrulata* a one-to-one subculturing had to be used. The top-layer agar method from Quispel and Burggraaf (1981) was tried for subculturing the Sp^+ isolates from *A. rugosa, A. glutinosa*, and *A. serrulata:* a bottom 1% W/V agar layer containing Qmod medium without peptone was covered with a 0.8% W/V agar layer containing peptone and 5 mg L^{-1} of lecithin, cooled to 40°C and inoculated with the fragmented colonies of presumptive *Frankia* isolates. The petri dishes were sealed and incubated at 27°C. SMA (Standard Methods Agar, BBL) plates and Qmod B tubes supplemented with 7% V/V FBS, whose purpose was to inhibit sporulaton (Lalonde and Calvert 1979), were also used for subculturing Sp^+ *Frankia* isolates. The method of Perradin (pers. comm.) was used for some Sp^+ strains. It involved the use of Qmod B tubes supplemented with benzoic acid to a final concentration of 10^{-3}M.

3.1.3 Infectivity tests

Infectivity tests in growth pouches were done according to Lalonde (1979b). The *A. glutinosa* seeds were surface sterilized by mechanical agitation in 30% H_2O_2 with a trace of Tween 20 (Sigma) as surfactant for 20 min. Five to ten seeds were placed in the trough of each growth pouch to which 15 mL of N-free Crone solution had been added. The pouches were placed in growth chambers at 15 000 lux for 16 hours, 22°C/17°C (day/night), for germination and growth. The seedlings were inoculated after the emergence of root hairs by placing a drop of phosphate-buffered saline (PBS)-washed and fragmented *Frankia* isolate on the roots. The pouches were fertilized bi-monthly with 1-10 ml of N-free Crone solution.

3.1.4 Acetylene reduction assay

The three month-old *A. glutinosa* seedlings from the infectivity tests in growth pouches were analysed for acetylene reduction and ethylene production according to Koch and Evans (1966). About 0.1 g fresh weight of nodules were excised and introduced into 20 X 150 mm glass test tubes sealed with rubber serum caps. Ten percent of the atmosphere was then replaced with acetylene and the tubes incubated for 1 hour at room temperature. Controls without C_2H_2 were also tested. Using a gas-tight glass syringe, a 25 μL sample was injected into a gas chromatograph (Hewlett-Packard, model HP5710A) equipped with FID and a 180 cm X 1 mm ID column filled with Porapak N, 80-100 mesh. The injection port, column, and detector temperatures were 100°C, 60°C, and 120°C respectively and the flow

rates of H_2, air, and N_2 carrier gas were 20, 200, and 30 mL min^{-1} respectively.

3.1.5 Lyophilization

The 3-5 week-old colonies were transferred with about 0.5 ml of the original Qmod B medium into 2 mL prescored ampoules (Wheaton Scientific, Millville, NJ). The ampoules were frozen at −85°C, dessicated in a Virtis freeze-dryer for 3 hours and vacuum-sealed. The validity of the technique was checked by subculturing samples of freeze-dried *Frankia* cells into tubes of new Qmod B medium.

3.2 Characterization of strains

The actinomycetes tested came from three sources. Strains of *Nocardia asteroides*, *Streptomyces griseus*, and *Actinoplanes philippinensis* were obtained from the American Type Cultures Collection (Rockville, Md) and bear the numbers # 19247, # 23345, and # 12427 respectively. The strain of *Streptomyces venezuelae* was kindly provided by Dr. Colin Stuttard (Dalhousie University medical school, Halifax, NS). All *Frankia* strains tested were isolated in our laboratory. All actinomycetes tested were grown on Qmod B medium.

3.2.1 Localization of sugars

To determine the localization of sugars in the *Frankia* cells (in other words, to define whether they are cytoplasmic or belong to the walls), colony clusters of isolate $ARgP5^{AG}$ were crushed by repeated passage through a French press, and filtered on 0.22 μm membranes. The two fractions were then dried under an air flux and tested by chromatography of their alditol acetate derivatives.

3.2.2 Alditol acetates derivatives

The analysis of sugars was done according to Mort and Lalonde (pers. comm.). The *Frankia* colony clusters from a single tube of Qmod B medium were harvested with Pasteur pipettes, washed in PBS, and centrifuged three times. A few drops of 10% V/V acetic acid were added to eliminate the $CaCO_3$ present in Qmod B medium. The colonies were then transferred to 1 mL reacti-vials (Wheaton Scientific, Millville, NJ) and 0.5 mL of 2N trifluoroacetic acid (TFA) was added. The vials were shut tight and heated to 121°C for 60 minutes, then left to cool to room temperature. The TFA was then evaporated under an air stream in a 40°C water bath. Fifty μL of a freshly prepared 20 mg/mL potassium borohydride in 3M NH_4OH solution were added to the vials to reduce the hydrolysates. If much bubbling occurred, 25 μL of potassium borohydride solution were added to neutralize the remaining TFA. The reaction was left to proceed for 1 hour at room temperature. A few drops of glacial acetic acid were added to terminate the reaction.

Then 500 μL of methanol were added to coevaporate with the borohydride under an air stream in a 40°C water bath. The coevaporation step with methanol was repeated five times or until the residue appeared frosty as opposed to transparent. The reduced sugars were acetylated by adding 50 μL of acetic anhydride and 50 μL of pyridine (previously dried over KOH pellets) and heating to 100°C for 15 minutes. The vials were cooled and then evaporated to dryness. If the process took too long, 100 μL of ethyl acetate (stored over grade stainfree molecular sieve 5A) were added. One hundred μL of ethyl acetate were added and evaporated. Then, 10-20 μL of ethyl acetate were added and 1-3 μL were injected into an HP 5830A gas liquid chromatograph equipped with a 180 cm long X 2 mm ID glass column filled with Gas Chrom Q coated with 0.3% OV-275 and 0.4% XF-1150 (or only with OV-275, 3%). The temperature program was: 140°C for four minutes, and then increasing temperature by 1°C min^{-1} up to 180°C. The injector and FID detector temperatures were 200°C and the gas flow rates for H_2, air, and the N_2 carrier gas were 20,300, and 28 mL min^{-1} respectively.

3.2.3 Trimethylsilyl derivatives

A few strains were also tested for sugars by their trimethylsilyl derivatives (Lalonde and Mort, pers. comm.). The cells were washed and hydrolyzed with TFA as previously. Then, 25 μL of a tri-Sil(R) solution (Pierce Chem. Co., Rockford, Ill.) were added to the vials with gentle shaking. After one hour at room temperature, 0.5 5 μL were injected into a gas liquid HP 5700 chromatograph equipped with a glass column 180 cm long x 2 mm ID filled with Chromosorb-W coated with SE-52, 3%. The temperature program was: initial temperature 140°C for 32 minutes, then 8°C min^{-1} up to 240°C. The injector and FID detector temperatures were 250°C and the gas flow rates of air, hydrogen, and helium carrier gas were 240,22, and 22.5 mL min^{-1} respectively.

3.3 Evaluation of strains

A subsample of strains isolated in the first part of this work were tested for nitrogen-fixing efficiency under optimal conditions. No criterion except that of availability of cultures was chosen. The efficiency tests in styroblocks were done according to Lalonde and Calvert (1979). *A. crispa* seeds from Val d'Or, P.Q. (78°W, 48°N) were soaked in 2°C water for 3-4 days and surface sterilized (see Section 3.4). The seeds were placed 4 or 5 at a time in the Turface-filled cavities of #8 styroblocks (Mansonville Plastics, Montréal) previously fertilized with N-free Crone solution, and germinated in transparent plastic bags under the same growth chamber conditions as those for the growth pouch infectivity tests.

After one month the N-starved seedling were thinned, placed one per cavity, and inoculated with a 1-month-old *Frankia* colony clusters at a density of one tube for 15 cavities. The isolates were washed in PBS and fragmented with a 2.5 cm 20G needle and a 3 ml plastic syringe. Five uninoculated controls were kept for each isolate tested.

After about three months of growth, the shoot height, fresh weight, oven-dry weight, and N-content (micro Kjeldahl) (Coles and Parks 1946) were measured.

3.4 Evaluation of sterilizing agents

A. crispa seeds were stored at 2°C in the dark for 8-10 months. Empty seeds were removed by flotation on a mixture of hexanes (density 0.662) (Wang, pers. comm.) before stratification in deionized water at 2°C for 5 days. The seeds were drained, rinsed with deionized water, and surface sterilized with one of the following:

(1) Commercial bleach (150 mL of 5.25% W/V NaClO) was poured into a 500 mL Erlenmeyer flask with 2 drops of tenfold diluted Tween 20 (Sigma). About 300 seeds were placed in the flask and mechanically agitated for different times.

(2) Hydrogen peroxide, bromine, and ethanol treatments were identical to that using NaClO except that 30% V/V hydrogen peroxide, 0.1% V/V bromine and 75% V/V ethanol respectively were used. A 1% W/V merthiolate (Thimerosal, Sigma) was used without addition of surfactant in the same way as with NaClO. About 300 seeds placed in a tea strainer were immersed in a petri dish containing 1% W/V aqueous solution of OsO_4 for the desired periods of time.

Seeds were drained after all treatments and rinsed by pouring 2 L of sterile distilled water slowly upon them. They were then plated 10 per plate on to Plate Count Agar (DIFCO) or 1.5% W/V sucrose agar. The Petri dishes were sealed with masking tape and seeds germinated at room temperature (24-26°C) under diffuse light. The seeds were examined after 5 days under a stereomicroscope (30X) and the number of axenic germinated seedlings (experiment # 1) or of axenic normal germinants (experiment # 2) recorded. A control germination on quartz sand gave 95% germinated seedlings.

4 RESULTS

4.1 Isolation of strains

In less than a year, 250 isolates were obtained from 33 provenances of *Alnus* spp., *M. gale* and *S. canadensis* (Table 5). Many were isolated from Quebec along the St. Lawrence seaway as shown in Figure 1. Following OsO_4 treatment, a whitish outgrowth occurred on the blackened nodule epidermis after an average of two weeks for Sp$^-$ and of 4 weeks for Sp$^+$ type strains. In the case of *A. rugosa*, *A. serrulata*, *A. viridis*, and *A. glutinosa* Sp$^+$, this adaptation could be much longer and isolates never became totally adapted to their new *in vitro* growth conditions. Many of the 50 tubes inoculated with crushed nodule lobes from one provenance were contaminated by bacteria, yeast, or fungi. Of the remaining uncontaminated tubes, from 0 to 100%, with an average of 80% (30 tubes), were found to contain presumptive *Frankia* isolates. This identification was based on observation under the interference light microscope of typical *Frankia* features, i.e. septate and branched hyphae, sporangia containing non-motile spores, and spherical pedunculate vesicles.

4.1.1 Subculture and growth

One month after the OsO_4 treatment of Sp$^-$ actinorhizae, the rubber-serum-cap sealed tubes were subcultured at a 1-in-4 dilution ratio (i.e. the original tube was divided into four new tubes). Upon the second subculturing, an acceleration in growth rate, coupled with an adaptation of the isolate to its new *in vitro* growth conditions from its former endophytic to its present free-living status, yielded colony clusters in 10 days that were ready for use in infectivity, effectivity, and sugars tests. In the case of some Sp$^-$ (ACN8, SNC10) and of all Sp$^+$ type isolates, the time period required for the emergence of an outgrowth important enough for subculture was two months or more, and the dilution ratio had to be reduced to 1 in 2.

Even then, in the case of some isolates (ARgP12, ARgP18, ASP4, and AGP8), growth did not resume and the isolates remained dormant. Using the double layer agar technique, we were able to obtain slow growth of the ASP4 isolates, but the technique did not work with the other Sp$^+$ isolates (ARgP12, ARgP18, and AGP8). Using FBS-supplemented Qmod tubes, we were able to obtain a very slow growth of ARgP18a, AGP8, and ARgP6 isolates (visible colonies after three months). The benzoic acid-supplemented Qmod B tubes permitted a very good growth of the AVP3n isolate with some morphological changes (colony density much reduced like that of Sp$^-$, very numerous small (5-15 μm) spherical sporangia instead of the bigger, rarer, less spherical sporangia associated with Qmod grown AVP3n).

In Qmod B standard tubes, it was found that the ACP17, ACN8, and MGP10 isolates showed a growth rate intermediary between the fast rate of Sp$^-$ type isolates and the very slow rate of the Sp$^+$ isolates.

ARgP6b and ArgP6d isolates, for instance, yielded colonies two months after isolation. These colonies were subcultured in standard Qmod B tubes with FBS and yielded rare, slow-growing, compact colonies. These were used for infectivity, effectivity, and sugars tests but a second subculturing failed and the isolates were considered dormant. The *A. crispa* seedlings which had been inocuated with the two isolates were treated with OsO_4 and the re-isolated *Frankia* strains ($ARgP6_b^{AC}$) showed no increase in growth rate. This indicated a stable genetic character.

Presumptive *Frankia* isolates were yielded by field-grown plant nodule clusters that were treated directly, up to leaf fall in November. No difference in the time delay necessary for emergence of the *Frankia* colonies, nor any difference in the *in vitro* growth rate or morphology, was noted between spring, summer, or fall isolates.

The $ARgP5^{AG}$ isolate obtained by the serial dilution technique (Lalonde 1979a) required two months for the appearance of colony clusters in Qmod B medium (Figure 2). When re-isolated by the OsO_4 technique from growthpouch-grown *A. crispa* seedlings ($ARgP5^{\underline{AG}\ AC}$), the time required for the appearance of colony clusters was reduced to two weeks (Figure 5).

4.1.2 Morphology of isolates

The isolates' morphology was, in general, as follows: a whitish mat covering the bottom of the tube; under the light microscope 100X objective, slender (0.5-1.0 μm in diameter), branched, and septate hyphae, sporangia of various shapes and sizes (5-100 μm) enclosing 0.2-1.0 μm non-motile spores, and occasional spherical pedunculate and septate vesicles (Figure 2). The isolates from Sp$^+$ nodules (Figure 3) produced much denser colonies, to the point where the colonies had to be disrupted before any observation under the light microscope could be made. The Sp$^+$ type isolates also produced more numerous and bigger sporangia (up to 150 μm) as compared to the usual (20-50 μm)

Table 5: List of provenances

Acronym	Isolates letters	Location	Soil type	Site type	Coordinates lat. N	Coordinates long. W	Source
A. crispa							
ACN3	a,b,..,j	Tadoussac	SS	W	48°10′	69°40′	PY
ACN4	b,c,..,j	Cap-aux-Oies	SL	RS	47°30′	70°15′	ML
ACN5	a,b,..,j	Lac Beauport	SS	RS	47°00′	71°15′	col.
ACN6	a,b,..,j	Gaspé	SS	RS	48°55′	64°35′	col.
ACN7	a,b,..,j	Percé	SL	DA	48°30′	64°10′	col.
ACN8	a,b,c,d	Baie St-Paul	SS	RS	47°25′	70°30′	col.
ACN10	a,b,..,t	Tadoussac	SS	W	48°10′	69°40′	YP
ACN11	a,b,..,s,u	Baie-St-Paul	SS	RS	47°25′	70°30′	col.
ACN12		Petite-Rivière	SS	RS	47°20′	70°35′	col.
ACN13	a,b,..,j	"	SS	RS	47°20′	70°35′	col.
ACN14	a,b,..,j	Tadoussac	SS	W	48°10′	69°40′	col.
TN15AC	a,b,..,e	Ste-Monique	SS	RS	48°45′	71°50′	ML
ACP17	a,b,..,j	Breakeyville	CL	DD	46°40′	71°15′	col.
TN16AC	a,b,..,h	Manitounuk	SS	W	55°40′	77°25′	AF
ACN20	a,b,..,j	Ste-Louise des-aulnaies	SS	RS	47°15′	70°05′	col.
ACN21	a,b,..,j	Port-au-persil	SS	RS	47°50′	69°50′	YP
A. rugosa							
ARgN4	a,b,..,j	Pointe-à la-garde	SS	DA	48°05′	67°25′	LS
ARgP5AG		Quebec city	SL	RS	46°45′	71°25′	col.
ARgP6	a,b,c,d	Tadoussac	CL	DD	48°10′	69°40′	col.
ARgN8	a,b,..,j	Sherbrooke	CL	DD	45°25′	71°55′	col.
ARgN9	a,b,..,e	St-Calixte	CL	RS	45°55′	73°50′	YP
ARgP12	a,b	St-Samuel	SS	RS	46°05′	72°15′	ML
ARgN14	a,b,..,j	St-Pierre	CL	W	46°15′	71°35′	ML
ARgN/P18	a-c(P) d-f(N)	Grand-Mère	SS	DA	46°35′	72°40′	ML
ARgN22	a,b,..,j	St-Magloire	SS	RS	46°35′	70°15′	ML
ARgN25	a,b,..,j	St-Omer	SS	RS	47°05′	69°40′	ML
ARgN30	a,b,c	Albanel	CL	DD	48°50′	72°20′	JD
A. viridis							
AVP3	a,b,p	Col du corbier (Alpes)	SS	RS	46°	4°(E)	AM
A. serrulata							
ASP4	a,b,..,e	Gainesville Fl. USA	CL	DD	29°	83°(W)	RJ
A. glutinosa							
AGP8	a,b,c,d	Vallée de l'orge (Paris)	CL	DD	49°	2°(E)	JM

Table 5: List of provenances (continued)

M. gale							
MGP10	a,b,..,j	Cap-aux-Oies	CL	W	47°30′	70°15′	ML
S. canadensis							
SCN9	a	Percé	SL	DA	48°30′	64°10′	col.
SCN10	a	Cap-aux-Oies	CL	W	47°30′	70°15′	ML

The acronym system is explained in Appendix A, the isolate letters give the number of isolate for each provenance; in the case of ARgN18 the Sp+ isolates are a,b,c and for Sp− they are d,e,f. The soil type was determined by hand (SS: sandy soil; SL: sandy loam; CL: clay loam). The site types are roadside (RS), wild area (W), disturbed area (DA), drainage ditch or river bank (DD). The sources are P. Younger (PY), Dr. M. Lalonde (ML), Y. Perradin (YP), Dr. J.-A. Fortin (AF), L. St-Laurent (LS), Dr. J. Dawson (JD), Dr. A. Moiroud (AM), R. Jobidon (RJ), and Dr. J. Mugnier (JM).

sporangia of Sp− type colonies (Figure 4). Some isolates (ACN8 and ARgN22) did not form normal sporangia but only very rare, tiny (5 μm) outgrowths that appeared to be aborted sporangia (Figure 6).

One of these isolates (ACN8a) was re-isolated after inoculation to *A. glutinosa* (ACN8 $^{AG}_{a}$)to check the genetic stability of this character, and no increase in sporangia number or size was noted between the two isolates.

The isolates SCN9a and SCN10a had morphological features typical of the *Elaeagnus* isolates (Figure 7), i.e. numerous vesicles, a dense matrix, and few, tiny (3-10 μm) sporangia, but without pigmentation as was the case with most other isolates from *Elaeagnus*. Their growth rates were also remarkably slow.

4.1.3 Infectivity

Of the 248 isolates tested, all were found to be infective, forming prenodules in 8-10 days after inoculation and one week later, nodules that permitted the *A. glutinosa* host to grow plants normally in N-free Crone solution. These nodules were sectioned and stained with cotton blue. These sections showed the same morphology as field-grown nodules. Control plants (uninoculated) were stunted and chlorotic (Figure 8b).

The SCN10a and SCN9a isolates were not tested for infectivity due to difficulties in subculturing them.

4.1.4 Microscopic observation of nodule sections

In the cotton blue-stained nodule sections, we observed *Frankia* hyphae and vesicles growing in the mid-cortical cells. Hyphae invaded the apical cells, forming a central network and, later, vesicles developed on the periphery of the cells. In some provenances, we observed inter- and intracellular sporangia (Sp+ type nodule) (Figure 8a).

The location of the Sp+ type provenances from the province of Quebec was generally in the St. Lawrence lowlands that are predominantly colonized by *A. rugosa*. In the highlands, where *A. crispa* is dominant, all *A. crispa* nodules were Sp− and the *A. rugosa* host, growing in its atypical dry upland habitat, had generally Sp−(ARgN14,22,25) except for some Sp+ (ARgP6). In the introgression zone, that is in the valley north of 47° (lat. N) and in the mountains south of the 47° (lat. N), both Sp+ and Sp− type were observed on *A. crispa* and *A. rugosa* host plants. At Cap-aux-Oies where *A. crispa*, *M. gale*, and *S. canadensis* were encountered, *A. crispa* and *S. canadensis* were Sp− and *M. gale* was Sp+.

4.1.5 Acetylene reduction assay

Nodule clusters formed by 14 different isolates chosen at random from the *A. crispa* styroblock or *A. glutinosa* growth-pouch-grown seedlings (Table 6) showed ethylene production in the range of 0.50 to 15 μmoles C_2H_4 $hour^{-1}$ g^{-1} oven-dry weight of nodules.

4.1.6 Lyophilization

All isolates which could be subcultured in Qmod B tubes were lyophilized in three replicates. Some strains such as ARgP5^{AG}, ACN8a, ACN10a, ANC14a, etc., which held a particular interest were lyophilized in larger quantities.

The slow growing Sp+ isolates such as ARgP6, ARgP12, AGP8 were not lyophilized but kept in sealed tubes and stored as "liquid cultures" in the collection.

4.2 Characterization of strains

All isolates that had been identified as *Frankia* on the basis of morphology and infectivity and tested for sugars were found to contain the sugar 2-0-methyl-D-mannose. All isolates listed in Table 7 were found to possess a sugar spectrum similar to that of strain TN15a^{AC}(Fig. 9) comprising rhamnose, ribose, xylose, mannose, galactose, glucose, and inositol besides 2-0-methyl-D-mannose.

Whole-cell hydrolysates of *Nocardia asteroides*, *Actinoplanes philippinensis*, *Streptomyces griseus* and *S. venezuelae* did not show 2-0-methyl-D-mannose peaks (Figure 9).

4.2.1 Localization of sugars

2-0-methyl-D-mannose did not go through the 0.22 μm membrane filter (Figure 10) presumably because it was not soluble. The relative proportions of other sugars changed also: ribose, galactose, and glucose were more prominent in the filtrate whereas mannose, xylose, and 2-0-methyl-D-mannose appeared in more important quantities in the non-soluble fraction.

4.2.2 Trimethylsilyl derivatives

Chromatography of trimethylsilyl derivatives of the ACN14a, SCN9a, and SCN10a isolates showed the presence of the same sugars and confirmed the identity of the 2-0-methyl-D-mannose peak as found by chromatography of alditol acetate derivatives.

Table 6. List of the 14 isolates tested as endophytes for acetylene reduction

TN15$^{AC}_{a}$	ARgP5^{AG}	ARgN14a	ARgN22d
TN15$^{AC}_{b}$	ARgN8a	ARgN18d	AVP3m
TN15$^{AC}_{c}$	ARgP12e	ARgN18f	AGP8a
ACP17b			MGP10a

Table 7. List of *Frankia* strains tested for sugars by their altidol acetate derivatives

ACN3a	ACN11a	TN15a^{AC}	ARgN14a
ACn5a	m	b	b
ACN6a	ACN12	ACP17a	ARgN18d
ACN7a	ACN13a	b	ARgN22a
ACN8a	b	TN18a^{AC}	d
b	c	ACN20b	ARgN25a
c	d	ACN21b	b
d	f	ARgN4a	ARgN30c
ACN10a	ACN14a(*)	ARgP5^{AG}	AVP3d
b	b	ARgP6a	f
c	c	b	m
d	d	ARgN8c	MGP10a
t	e	ARgN9c	SCN9a(*)
			SCN10a(*)

(*) Strains tested also by derivation into trimethylsilyl.

4.3 Evaluation of strains

Forty isolates were tested in three successive tests in styroblocks. The parameters measured, height (Table 8), fresh weight (Table 9), oven-dry weight and N-content, were found to be well correlated (e.g. R = 0.94 between height and N-content averages of each isolate). The three tests were conducted under different growth conditions, resulting in different overall parameter averages (Figure 11). To make the tests comparable, the data were transformed in percentages of the most effective in each test, yielding two clusters, one of Sp^- and one of Sp^+ type isolates (Figure 12). On these transformed data, a Student's t-test on linearized % (arcsin $\sqrt{x}$) was performed for both the N-content and height averages.

Figures 11 and 12 show that Sp^- and Sp^+ type isolates were significantly different at the 99% level and

Table 8: Height of seedlings

Test # 1			*Test # 2*			*Test # 3*		
ACN10a	28.1 – 3.3	a	ACN14a	22.1 – 3.2	a	ARgN14a	11.1 – 3.3	bcd
b	27.7 – 4.4	a	b	19.9 – 3.9	ab	b	14.2 – 3.3	a
c	27.0 – 5.1	a	c	23.1 – 4.6	a	ARgN22a	12.9 – 2.5	ab
d	28.0 – 5.3	a	d	21.1 – 4.2	a	d	12.1 – 2.6	abc
ACN11a	34.7 – 3.6	ab	e	21.6 – 3.4	a	ARgN18d	10.1 – 2.0	cde
b	21.9 – 6.6	bcd	ACN13A	19.6 – 4.4	ab	f	12.7 – 3.1	ab
ARgPt^{AG}	23.2 – 5.2	d	b	20.7 – 5.5	a	TN15$_b^{AC}$	12.6 – 2.7	ab
ACN8a	19.0 – 5.1	c	c	20.5 – 4.6	a	d	11.1 – 2.7	bcd
b	21.7 – 4.2	bc	d	20.7 – 2.6	a	ACP17a	9.8 – 2.0	de
c	22.3 – 4.9	bc	f	20.0 – 3.2	ab	b	9.1 – 2.3	de
AVP3d	13.2 – 9.9	cd	ACN12	21.8 – 2.4	a	AVP3m	8.9 – 2.3	e
f	17.4 – 5.2	d	ACN11m	21.1 – 4.6	a	MGP10a	6.3 – 2.6	e
control	1.5 – 0.5	c	ACN10t	21.9 – 3.9	a			
			ACN8d	17.5 – 4.5	b			
			ARgP6b	13.9 – 6.1	c			
			d	18.4 – 3.8	b			

Table 9: Fresh weight of seedlings

Test # 1			*Test # 2*			*Test # 3*		
ACN10a	9.1 – 2.4	a	ACN14a	5.7 – 1.3	a	ARgN14a	1.6 – 0.7	cd
b	6.8 – 2.4	b	b	5.0 – 1.4	ab	b	2.1 – 0.8	abc
c	9.1 – 2.4	a	c	5.7 – 1.6	a	ARgN22a	2.5 – 0.8	ab
d	8.0 – 2.4	ab	d	5.4 – 1.5	a	d	2.2 – 0.8	abc
ACN11a	6.7 – 1.9	b	e	5.0 – 1.9	ab	ARgN18d	1.8 – 0.5	cd
b	4.5 – 1.9	c	ACN13a	4.8 – 1.9	ab	f	2.0 – 0.7	bcd
ARgP5^{AG}	6.4 – 2.2	b	b	4.5 – 2.3	ab	TN15$_b^{AC}$	2.5 – 0.7	a
ACN8a	5.7 – 2.2	bc	c	4.8 – 2.0	ab	d	1.9 – 0.7	cd
b	5.9 – 2.1	bc	d	4.9 – 1.4	ab	ACP17a	1.7 – 0.6	cd
c	5.0 – 2.5	bcd	f	5.3 – 1.4	a	b	1.4 – 0.5	cd
AVP3d	3.2 – 3.7	d	ACN12	4.7 – 1.3	ab	AVP3m	1.3 – 0.5	cd
f	4.3 – 2.2	cd	ACN11m	4.8 – 1.4	ab	MGP10a	1.1 – 0.5	d
control	0.3 – 0.2	e	ACN10t	5.1 – 1.6	a			
			ACN8d	3.5 – 1.7	bc			
			ARgP6b	2.3 – 1.9	c			
			d	3.7 – 1.2	bc			

that the Sp⁻ type isolates ACN8a, b, c, and d, ACN11b, ARgN18a, TN15a^{AC}, and ARgN14d were found within the Sp⁺ type isolate cluster. The variations within and between provenances were generally not significant except for ACN11, ARgP6, ARgN14 and ARgN18 provenances (see Tables 8, 9). Isolates from provenances ACN10, ACN8, AVP3, ACN13, ACN14, ARgN22, TN15 and ACP17 were not significantly different. From these tables (8, 9) it is clear that Sp⁻ type strains (ACN10, ACN14, and ARgN14) were always more effective than Sp⁺ strains (AVP3, ARgP6, and MGP10).

The efficiency tests showed that, generally, no significant differences existed between height of isolates from one provenance except for three *A. rugosa* provenances (ARgP6, ARgN14, and ARgN18). The fresh weight parameter showed slightly different results (significant differences at the 95% level were observed with ACN10, ACN11, and TN15^{AC} but not with ARgN14, ARgN18, and ARgP6). Considering that fresh weight is a much less stable parameter, the height results were preferred.

Variations between provenances were significant in some cases but the important source of difference was the Sp⁺ type and Sp⁻ type endophytic character (Figure 12). The most effective inoculants, as determined by the height parameter, in the three tests were of the Sp⁻ type (ACN10, ACN14, and ARgN14a) and the least effective were of the Sp⁺ types AVP3, ARgP6, and MGP10 (Figures 13, 14, 15).

4.4 Evaluation of surface sterilizing agents

In the first experiment, 5.25% NaClO, 1% OsO_4, 30% H_2O_2, 1% merthiolate, and 75% ethanol were used for periods ranging from 5 to 125 min (Figure 16a). Merthiolate, ethanol, and OsO_4 treatments did not give satisfactory results because they never resulted in more than 65% axenic germinants. Even if the merthiolate treatment yielded up to 63% axenic seedlings after 20 min, a large number had stunted radicles. NaClO was found to be efficient but required up to 80 minutes to yield more than 80% axenic germinated seedlings. For periods longer than 40 minutes in NaClO, high percentages of malformed seedlings were observed. Hydrogen peroxide yielded more than 80% sterile germinated seeds and, after 25 minutes, more than 80% of the seeds were still axenic and normal.

Contaminants obtained on sucrose agar were identical to those obtained on Plate Count Agar except that the growth of contaminants (fungi and bacteria) was much faster on Plate Count Agar. Sometimes this made it impossible to detect germinations. The radicle growth was also much reduced on Plate Count Agar and its use was discontinued for experiment # 2.

In a second experiment (Figure 16b), 0.1% Br, 5.25% NaClO, and 30% H_2O_2 were used. Only axenic normal seedlings were recorded.

Bromine was found to be very efficient, requiring a short treatment time to produce a maximum number of usable seedlings. Treatment time was critical since after 10 minutes many seedlings were abnormal. Sodium hypochlorite was also very efficient but required 30 min to yield more than 80% axenic undeformed germinants. Hydrogen peroxide gave the highest percentage of axenic normal seedlings and prolonged treatment of up to 30 min did not affect radicle growth.

The curves for H_2O_2 (Figures 16a and b) are comparable whereas those for NaClO are different, the values after 30 min being 60% and 90% respectively.

5 DISCUSSION

5.1 Isolation of strains

5.1.1 Morphology and infectivity

The morphology of the *Frankia* isolates was similar to that described by Callaham et al. (1978), Berry and Torrey (1979), Lechevalier and Lechevalier (1979), Baker and Torrey (1979), Burggraaf et al. (1981) and Lalonde et al. (1981), except that typical *Frankia* vesicles described by Lalonde and Calvert (1979) were found in all isolates, in addition to the typical septate branched hyphae and sporangia.

The isolates obtained were all infective, forming nodules as described by Angulo Carmona (1974). The morphology of the nodules was similar to those from which *Frankia* had been isolated, fulfilling Koch's postulates. Other workers have not always been successful in re-infectivity tests. For instance, the two *Frankia* isolates from *Casuarina* nodules described by Gauthier et al., (1981) were not found to be infective on *Casuarina* seedlings. The isolates obtained by Callaham et al. (1978), Berry and Torrey (1979), Baker and Torrey (1980) were all infective, although one isolate from *Elaeagnus* was definitely classified as non-infective on the same host plant (Baker et al. 1980). Some isolates defined as *Streptomyces* may have been *in vitro* non-sporulating and non-infective *Frankia* (Berry and Torrey 1979). Burggraaf et al., (1981) described some of their isolates as non-infective after marked changes in rate of growth and growth requirements.

It is possible that some disruptive isolation techniques destroy most of the *Frankia* hyphae present in the nodule. This may cause not only a much longer adaptation period, but may also oblige the cells to undergo more divisions than in the OsO_4 method in order to achieve the minimum number of cells required for subculturing. Greater cell division could increase the probability of mutations such as those causing non-nodulation (Burggraaf et al. 1981).

The morphology of the nodular endophyte of host plants of the genus *Alnus* was similar to that described in Lalonde and Fortin (1973), and Lalonde and Knowles (1975b). The morphology of the endophyte of *Myrica gale* nodules was similar to that described by Bowes et al. (1977) and by Benson and Eveleigh (1979). The morphology of the nodular endophyte of *Shepherdia canadensis* was similar to that of *Hippophaë rhamnoides* described by Gatner and Gardner (1970).

5.1.2 Sp^+/Sp^- distributions

The presence of spores and sporangia (Van Dijk and Merkus 1976) was noted in some nodules of *Alnus* and *Myrica* spp. but contrary to Kappel and Wartenberg (1958) and Van Dijk (1978) who could not discern any relationship between the presence or absence of sporangia and ecological factors, we found a pattern of distribution where Sp^+ type endophytes were predominantly associated with *A. rugosa* in the fertile lowlands and Sp^- in the dry uplands. We, therefore, suggest that the situation found in Quebec could shed some light on the ecological significance of the two types concept (Van Dijk 1978). *A. rugosa* and *A. crispa* occur in an area in the St. Lawrence Valley that overlap north of 47°N (Figure 1). South of that imaginary line, scattered clumps of *A. crispa* seedlings are encountered, usually in wet lowland sites that are normal habitats of *A. rugosa*. North of the line (Figure 1), *A. crispa* is the dominant actinorhizal species, but *A. rugosa* thrives in dry upland habitats usually dominated by *A. rugosa* elsewhere. The *Alnus* genus has been evolving in North America since the latest glaciation (Daly 1966) and evidence from pollen studies (Richard 1977) and the presence of varying disjunct populations of *A. crispa* in the southern Appalachians (Furlow 1979) suggest that the range of this species had a more southern limit than it has today.

Since *A. crispa* is typically in symbiosis with Sp^- endophytes and *A. rugosa* with Sp^+ type endophytes, it was surprising to see that almost all *A. rugosa* plants growing in atypical habitats (dry upland sites north of the 47°) were inhabited by Sp^- type *Frankia*. *A. crispa* growing south of their normal dominant range hosted mostly Sp^- type *Frankia*. A few exceptions such as ACP17 exist. The pattern suggests that *A. rugosa* moved northward faster than its usual symbiont, and that it was colonized by atypical Sp^- type *Frankia* left behind after having inhabited its usual host *A. crispa*.

The distribution pattern of Sp^+ and Sp^- types of *Frankia* could also have been brought about by ecological adaptation not only to the host plants *A. crispa* and *A. rugosa*, but also to soil nitrogen (high in wet lowlands, low in dry uplands), soil pO_2, pH or other factors. Such a pattern of endophyte distribution had also been encountered in *Ceanothus velutinus* by Wollum et al. (1968) who hypothesized that through selection, two populations of *Frankia* would develop showing differing specificities.

The presence of sporangia in nodules from other host plants from which isolation was performed was not investigated beyond the determination of the type of the particular nodules we worked with.

The 2-week time required for the appearance of presumptive *Frankia* isolates after isolation with the OsO_4 method contrasts with the 2-month period encountered when other, more disruptive, techniques such as serial dilution (for $ARgP5^{AG}$), sucrose density centrifugation (Baker and Torrey 1979), or selective incubation (Quispel and Burggraaf 1981) are used. The isolation procedure that was employed in this study was always successful with all Sp− type endophytes. However, it was less effective for the Sp+ type isolates.

One reason could be the fact that *A. rugosa*, *A. glutinosa*, and *A. serrulata* contain greater amounts of phenols than *A. crispa* and *A. viridis* nodules (pers. obs.). Also the difference in growth requirements of Sp+ type among isolates from different host plants (Burggraaf et al. 1981) could be responsible for growth delays. The *in vitro* growth rate of ACP17, AVP3, and MGP10 isolates was lower than that of the Sp− type isolates but higher than that of the other Sp+ type isolates coming from *A. rugosa*, *A. glutinosa*, and *A. serrulata*. This finding is similar to that of Burggraaf et al. (1981) who found that *Myrica gale* Sp+ type isolates had a less stringent growth requirements than *A. glutinosa* Sp+ type isolates.

The fact that ARgP6b did not grow faster after re-isolation from *A. crispa* ($ARgP6b^{AC}$) may mean either that a genetic determinant is involved or else that it is a quite stable phenotypic character. This is often the case with actinomycetes whose morphology is influenced for many subcultures by the previous history of the culture (Waksman 1959). We found no significant change after up to ten re-isolations of $ACN1^{AG}$ which would support the genetic determinant hypothesis.

5.1.3 Growth conditions

Clearly the growth conditions used by us and most other researchers were adequate for isolates from Sp− nodules. The result was variable for Sp+ spores. We tried a few different procedures such as the double agar layer (Burggraaf et al. 1981), the Qmod B-FBS (Lalonde and Calvert 1979) or the Qmod-benzoic acid (Perradin, pers. comm.) techniques and found that each of these worked in some cases but not all. More testing is therefore required to find growth conditions that are well suited for all *Frankia*, especially the Sp+ isolates. Obviously the best approach is to try to reproduce conditions in the nodules. Some work in this area has recently been initiated (Gardner et al. 1979, Knights et al. 1981, Perrot-Rechenmann et al. 1981). The appearance of *Frankia* hyphae and spores on the blackened epidermal surface of Sp+ nodules and failure to subculture these isolates (ARgP6, ARgP12, AGP8) have not been mentioned by other authors. This perhaps is indication that both medium composition and growth conditions have prevented the isolation in pure culture of many *Frankia* strains.

5.2 Characterization of isolates

Sugar analysis of nodule isolates has demonstrated the value of the test to confirm the identity of strains presumed to be *Frankia* on the basis of morphology and infectivity on a compatible host-plant. The eight typical sugars (Figure 9) were found in all isolates tested (Table 7) in similar proportions, independently of the host-plant origin or of the "sporulation type". This fact is indicative of the high structural homogeneity that exists in the genus *Frankia*.

The sugar spectrum also gives important information on cell wall structure (Brock 1974). It is in the cell wall that the sugar 2-0-methyl-D-mannose is found.

Although this sugar seems characteristic of *Alnus* spp., it should not be taken for granted, because nowhere is it stated in the literature as not having been found in the sugar spectra of other organisms. The equipment needed to identify an unknown sugar such as 2-0-methyl-D-mannose is costly and is not readily available; to use it only to identify a minor peak would not be worthwhile. Analysis of the whole sugar spectrum is more reliable an indicator than the identification of one peak.

The alditol acetate and trimethylsilyl derivatives can both permit the detection of eight typical sugars, but the simplicity of trimethysilyl derivative preparation is compensated by difficulties associated with the presence of two peaks per sugar. However, their coordinated use is strongly recommended, especially in case of doubt, as it was for the SCN9a and SCN10a isolates whose morphological features were not typical.

It is important to include in any work on bacterial taxonomy a biochemical test to confirm the results of the morphological or physiological tests. The analysis of the sugar spectrum is useful and can fulfill this role in the genus *Frankia*.

5.3 Evaluation of isolates

The existence of a significant difference between the Sp+ type and the Sp− type isolates had been suggested by Hall et al. (1979) who used crushed-nodule inocula of Sp+ and Sp− type on *A. rubra* and *A. glutinosa*. Maynard (1980) also found such a difference between Sp+ and Sp− crushed-nodule inocula on *A.*

glutinosa, as well as a market effect of the inoculum's previous history of efficiency. For instance, the geographical closeness of an inoculum to the host improved all parameters measured.

The character of Sp+ and Sp− types and the previous history of the isolates would then be the most important source of variation between isolates. The major difference due to the Sp+ and Sp− types and the minor differences noted between isolates of one provenance and between provenances validate our procedure of isolating up to ten isolates from one provenance and from many provenances. This procedure has been made possible by the OsO_4 isolation technique (Lalonde et al., 1981).

The isolates grouped in the upper right-hand part of the Sp− cluster (Figure 7) represent the best strains for mass inoculation of *A. crispa* for dry nitrogen-poor soils. In general, the conclusion of Maynard (1980) concerning the higher efficiency of Sp− type strains over Sp+ is similar to what we have found and means that generally Sp− type isolates are to be preferred to Sp+. Nevertheless, Sp+ certainly have an ecological advantage over Sp− and may be preferable under conditions where long and high percent of survival of the inoculum is critical or where the nitrogen content of the soil is high. Further research is needed to establish the ecological value of Sp+ type strains. The situation in Quebec could throw light on this.

The nodulation delay noted by Lalonde (1977) using crushed-nodule inocula of different alder species was not evident except in the case of MGP10a, and that resulted in a lower efficiency.

Three objectives had been set before the work reported here began: 1) isolation of a large number of *Frankia* strains and characterization of these isolates on the basis of morphology and infectivity, 2) confirming the identity of strains by analysis of the sugars spectrum containing, among others, 2-0-methyl-D-mannose and, 3) evaluation of the nitrogen-fixing efficiency of a subsample of isolates in symbiosis with *A. crispa*.

Two hundred and fifty *Frankia* strains were isolated from *A. crispa*, *A. rugosa*, *A. glutinosa*, *A. viridis*, *A. serrulata*, *Myrica gale* and *Shepherdia canadensis* and have been deposited in the Laval University collection (Faculté de Foresterie et de Géodésie, Dr. M. Lalonde).

The isolation method used was based on the utilization of OsO_4, an electron microscopy fixative, whose rapid and efficient action has made simple and reproducible the isolation of a large number of strains from different provenances. The OsO_4 treatment has also made possible the isolation of mutant strains never before described in the literature such as the *in vitro* non-sporulating ACN8 and ARgN22 isolates, and also of the Sp+ strains AGP8, ASN4, ARgP12, and ARgP18 that are difficult to isolate and cultivate with simple, classical methods (Quispel and Burggraaf 1981).

These strains, which were characterized according to their morphology, infectivity on a compatible host-plant and their *in vitro* growth rate, represent a good part of the natural spectrum of *Frankia* strains, endophytes of *A. crispa* and *A. rugosa* in Quebec. Most of these strains were similar in all respects to the CpI1 (Callaham et al. 1978) and AvcI1 strains (Baker and Torrey 1980) and some Sp+ type strains were similar to isolates from *A. glutinosa* and *M. gale* (Burggraaf et al. 1981). A few peculiar isolates were obtained that had never been mentioned in the literature before, as was to be expected because the isolation work reported herein is probably the most comprehensive ever achieved.

Characterization of the isolates as *Frankia* strains was based primarily on morphology, a straightforward criterion. Then came the infectivity tests on a compatible host-plant, a criterion used for confirmation. In case of doubt, and in a general way, to establish the value of the test, an analysis of sugars of whole-cell hydrolysates of presumptive *Frankia* isolates was performed to detect the presence of eight sugars typical of *Frankia* cells: rhamnose, ribose, xylose, 2-0-methyl-D-mannose, mannose, galactose, glucose, and inositol (Lalonde and Mort, pers. comm.). The most important but not the only relevant sugar was 2-0-methyl-D-mannose, a sugar marker of *Frankia*.

The three criteria mentioned above make up together a valid, simple, and coherent set that constitutes a reliable marker for *Frankia*.

An important homogeneity in the infectivity and sugar spectrum characteristic of the isolates was noted: all were infective and all had similar concentrations of the eight sugars mentioned before. However, the morphological characteristics and the *in vitro* growth rates varied, mostly according to the sporulation type to which the isolates belonged (Sp+ or Sp−).

The nitrogen-fixing efficiency of a subsample of strains was tested in growth chambers in three successive tests. Strains from the same provenance had similar efficiencies in general, but the few exceptions found validated our procedure. This consisted of isolating and subculturing up to 10 strains from each provenance using the OsO_4 technique. The most important source of variation in nitrogen-fixing efficiency, as measured by parameters of growth in conditions of total absence of fixed nitrogen, was the differentiation of isolates to the Sp+ or Sp− type groups. Sp+ isolates had a nitrogen-fixing efficiency that was around 70% of that of Sp− type (Table 4).

This study clearly demonstrated that Sp+ and Sp− type isolates are different according to morphological characteristics, growth rates, and nitrogen-fixing efficiencies. *In vitro* morphological differences are herein noted for the first time. *In vivo* morphological differences had been discussed by Van Dijk (1978), differences in *in vitro* growth rate and growth requirements had been noted by Burggraaf et al. (1981), and differences in nitrogen-fixing efficiencies had been investigated by Hall et al. (1979), and by Maynard (1981).

For these reasons, it is suggested that "sporulation type" be retained as a valid basis for species definition in the genus *Frankia*. Moreover, the terms "Sp+" and "Sp−" that refer exclusively to the endophyte should be replaced by the expresions "Type P" and "Type N" to designate respectively Sp+ and Sp− type *Frankia* isolates, both in their endophytic and their (*in vitro*) pure culture conditions.

5.4 Evaluation of sterilizing agents

Only 30% H_2O_2, 5% NaClO and 0.1% bromine can be recommended for surface sterilization of *A. crispa* seeds. Ethanol and OsO_4 did not give satisfactory percentages of surface sterilization (over 80%) without affecting the vitality of the seedlings. Merthiolate yielded up to 63% germinated seedlings without bacterial or fungal growth, but it is described as a bacteriostatic agent (Brewer 1957) and bactericidal only after long exposures, by which time the seedlings are adversely affected. The use of merthiolate (Jobidon and Thibault 1980) may be satisfactory to retard growth of contaminants and give a good start to the seedlings, but its use can certainly not be recommended when axenic host plants are required for nodulation studies on "helpers" (Knowlton et al. 1979).

Bromine was satisfactory but treatment time was found to be critical and must not exceed 5 min. The main disadvantages of bromine and OsO_4 are their volatility and human toxicity.

NaClO could have also yielded a high percentage of usable seedlings but the optimum exposure time was critical and, also, variable. That was due to the mode of action of NaClO which digests its way through the teguments. Slight differences in experimental parameters such as temperature may have been responsible for the erratic results obtained with NaClO. A longer exposure time than for H_2O_2 treatment was necessary to obtain a large number of axenic seedlings but malformed seedlings were observed at high frequencies if treatment was longer than 30 minutes.

These tests, conducted with all surface-sterilizing agents mentioned in the literature on alder seeds, are the first systematic study. It appears to us that the methods described here are valid and should be applied when one starts to work on a new species.

Stabilized 30% H_2O_2 with Tween 20 as surfactant, applied at room temperature during 5-30 minutes as recommended by Neal et al., (1967), was found to be the most reliable surface sterilizing agent for *A. crispa* seeds. It may yield up to 90% usable seedlings, i.e. axenic and normal plantlets. Hydrogen peroxide is an organically catabolizable compound that does not leave toxic residues.

6 CONCLUSIONS

A large-scale program has resulted in the isolation of 250 *Frankia* strains from *Alnus crispa, A. rugosa, A. viridis, A. glutinosa, A. serrulata, Myrica gale* and *Shepherdia canadensis*, representing a large part of the spectrum of natural variation found n *Frankia* endophytes of *A. crispa* and *A. rugosa*. It must be pointed out that even though the OsO_4 treatment is an easy-to-use, reliable, and rapid isolation method and has resulted for the first time in isolating *in vitro* non-sporulating mutants, the growth techniques and conditions used are not adequate for some Sp^+ strains. The difficulties encountered in isolating Sp^+ strains in pure culture were much more pronounced in the case of *A. rugosa, A. serrulata*, and *A. glutinosa* than with *A. viridis, A. crispa*, and *M. gale*, pointing to an influence of the host plant. This influence could be due to phenolic compounds which appeared to be present in greater amounts in *A. rugosa, A. serrulata*, and *A. glutinosa* than in *A. crispa, A. viridis*, and *M. gale*. The intensity of reddening of the PVPPBS crushing solution is a reliable indicator of the level of phenolic compounds. Most isolates fell into two groups based on their morphology: the Sp^+ and the Sp^- groups with differences in the number and size of sporangia and compactness of colonies. Some others had only tiny and rare sporangia. None were pigmented. All isolates tested were found infective on a compatible host plant.

The use of a biochemical test is always useful in taxonomy. In our study this test was importants in confirming the presumptive indicators based on morphology or infectivity. It was found that the chromatographic analysis of sugars of whole cell hydrolysates of presumptive *Frankia* isolates could either confirm or reject identification based on other criteria. The *Frankia* cell can be defined as having the following eight sugars: rhamnose, xylose, 2-0-methyl-D-mannose, mannose, galactose, glucose and inositol. One of these, 2-0-methyl-D-mannose, can be defined as a marker of *Frankia*. It must be pointed out, however, that not all bacteria genera have been tested and the non-appearance of 2-0-methyl-D-mannose in the taxonomic literature is not proof that it does not exist somewhere else. So, the analysis of the sugar spectrum, more than the detection of 2-0-methyl-D-mannose, is a useful tool to positively identify an isolate as belonging to the genus *Frankia*. But sugar analysis cannot give information such as sporulation type or host-plant origin because all isolates tested had similar sugar spectra.

The evaluation of the nitrogen-fixing efficiency of strains was made by the measurement of the biomass produced by *A. crispa* seedlings grown under conditions of total absence of fixed nitrogen. The most important source of variation was the sporulation type. The Sp^+ cluster was shown to have an efficiency of about 70% of that of the Sp^- cluster of strains. The host-plant origin did not produce significant differences. Significant differences were sometimes encountered between isolates from a single provenance, validating our procedure of isolating up to 10 isolates per provenance.

The sporulation type influences the *in vitro* morphology and rate of growth as well as the *in vivo* nitrogen-fixing efficiency. We therefore suggest that sporulation type be used as a valid basis for species definition in the genus *Frankia*.

Stabilized hydrogen peroxide (30% V/V) is recommended as reliable agent for sterilizing the surface of *Alnus crispa* seeds for axenic seedlings.

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APPENDIX A

Following is a description of the acronyms used in this report and a list of actinorhizal species known to date (after Akkermans and Houwers 1979).

Signification of Acronyms

The *Frankia* isolates were given an acronym according to the system of Lalonde et al. (1981.) First, one letter or a group of letters to describe the host species (e.g. AC for *A. crispa*, ARg for *A. rugosa*, T for isolates obtained from growth-pouch-cultivated seedlings inoculated with *Frankia*-containing soils), then a letter to define whether or not sporangia were observed in the nodule from which isolation was performed (e.g. AC<u>N</u>7a is an isolate obtained from a non-sporulating endophyte, and ARgN/P18 is a provenance from which both types of isolates were obtained). Then comes an arbitrary number given to each provenance and a letter to identify the different isolates from one provenance (ACN14a, ACN14b, ..., ACN14j). Sometimes at the upper right corner are placed letters to describe the fact that the strain was reisolated from a different host species (ACN8a^{AG} is an ACN8a isolate obtained initially from *A. crispa* and then reisolated from *A. glutinosa*). When those letters are underlined, it means that the host plant was inoculated with a crushed-nodule inoculum (Lalonde 1979a) and isolation then performed without a previous direct isolation (ARgP5$^{\underline{AG}}$).

Tn15a$^{\underline{AC}}$ for instance is the isolate "a" from provenance "15", from which a dried soil sample ("T") was inoculated to *A. crispa* (AC) and found to produce Sp$^-$ nodules (N).

List of genera and species of actinorhizal plants and proposed acronyms

ALNUS spp.				
	accuminata	AA	*nepalensis*	AN
	cordata	ACo	*nitida*	ANi
	cremastogyne	ACr	*oblongifolia*	AO
	crispa	AC	*orientalis*	AOr
	fauriei	AFa	*pendula*	AP
	firma	AF	*rhombifolia*	AR
	fermosana	AFo	*rubra*	ARb
	fructicosa	AFu	*rugosa*	ARg
	glutinosa	AG	*serrulata*	AS
	hirsuta	AH	*serrulatoides*	ASe
	incana	AI	*siberica*	ASb
	inokumai	AIn	*sieboldiana*	ASo
	japonica	AJ	*sinuata*	ASi
	jorullensis	AJo	*subcordata*	ASu
	lanata	AL	*tenuifolia*	AT
	maritima	AM	*tinctoria*	ATi
	matsumurae	AMa	*trabeculosa*	ATr
	maximowiczii	AMi	*undulata*	AU
	mayrii	AMy	*viridis*	AV
	multinervis	AMu		
CASUARINA spp.				
	cunninghamiana	CaC	*muricata*	CaMu
	decussata	CaD	*nana*	CaN
	deplancheii	CaDe	*nodiflora*	CaNo
	distyla	CaDi	*papuana*	CaP
	equisetifolia	CaE	*pusilla*	CaPu
	fraseriana	CaF	*quadrivalvis*	CaQ
	glauca	CaG	*rigida*	CaR
	huegeliana	CaH	*rumphiana*	CaRu
	lepidophloia	CaL	*stricta*	CaS
	littoralis	CaLi	*sumatrana*	CaSu
	montana	CaM	*tenuissima*	CaT
	muellerana	CaMe	*torulosa*	CaTo
CEANOTHUS spp.				
	americanus	CeA	*diversifolius*	CeDf
	azureus	CeAz	*fendleri*	CeF
	cordulatus	CeC	*foliosus*	CeFo
	crassifolius	CeCr	*fresnensis*	CeFr
	cuneatus	CeCu	*glabra*	CeG
	delilianus	CeD	*gloriosus*	CeGo
	divaricatus	CeDa	*greggii*	CeGe

List of genera and species of actinorhizal plants and proposed acronyms (continued)

	grieus	CeGi	*oliganthus*	CeO
	impressus	CeIm	*ovatus*	CeOv
	incana	CeIn	*parvifolius*	CeP
	intergerrimus	CeI	*prostratus*	CePr
	intermedius	CeId	*rigidus*	CeR
	jepsonii	CeJ	*sanguineus*	CeS
	leucodermis	CeL	*sorediatus*	CeSo
	microphyllus	CeM	*thyrsiflorus*	CeT
			velutinus	CeV
CERCOCARPUS spp.				
	betuloides	CrB		
	ledifolius	CrL		
	montanus	CrM		
	paucidentatus	CrP		
CHAMAEBATIA spp.				
	foliolosa	ChF		
COLLETIA				
	cruciata	CiC		
	paradoxa	CiP		
COMPTONIA spp.				
	peregrina	Cp		
CORIARIA spp.				
	angustissima	CoA	*nepalensis*	CoN
	arborea	CoAr	*plumosa*	CoP
	intermedia	CoI	*pottsiana*	CoPo
	japonica	CoJ	*pteridoides*	CoPt
	kingiana	CoK	*sarmentosa*	CoS
	lurida	CoL	*thymifolia*	CoT
	myrtifolia	CoM		
COWANIA spp.				
	mexicana var. *stansburiana*			CwM
DATISCA spp.				
	canabina	DaC		
	glomerata	DaG		

List of genera and species of actinorhizal plants and proposed acronyms (continued)

DISCARIA spp.				
	tournatou	DiT		
DRYAS spp.				
Dryas	*drummondii*	DyD		
	integrifolia	DyI		
ELAEAGNUS spp.				
	angustifolia	EA	*longipes*	ELo
	argentea	EAr	*macrophylla*	Em
	commutata	EC	*matsunoana*	EMa
	conferta	ECo	*montana*	EMo
	edulis	EE	*multiflora*	EMu
	formosana	EF	*murakamiana*	EMk
	glabra	EG	*numajiriensis*	EN
	latifolia	EL	*pungens*	EP
	longipines	ELo	*rhamnoides*	ER
	macrophylla	Em	*umbellata*	EU
			yoshinoi	EY
HIPPOPHAË spp.				
	rhamnoides	HR		
MYRICA spp.				
	adenophora	MA	*humidis*	MJ
	brevifolia	MB	*integra*	MI
	californica	MCa	*javanica*	MJ
	cerifera	MC	*kandtiana*	MK
	conifera	MCo	*kraussiana*	MKr
	cordifolia	MCd	*microbractea*	MM
	diversifolia	MD	*pensylvanica*	MP
	esculenta	ME	*pilulifera*	MPi
	faya	MF	*pubescens*	MPu
	gale	MG	*quercifiora*	MQ
	rubra	MR		
	salicifolia	MS		
	sapida	MSa		
	serrata	MSe		
PURSHIA spp.				
	glandulosa	PG		
	tridentata	PT		

List of genera and species of actinorhizal plants and proposed acronyms (continued)

RUBUS spp.		
	ellipticus	RE
SHEPHERDIA spp.		
	argentea	SA
	canadensis	SC

APPENDIX B

Figures

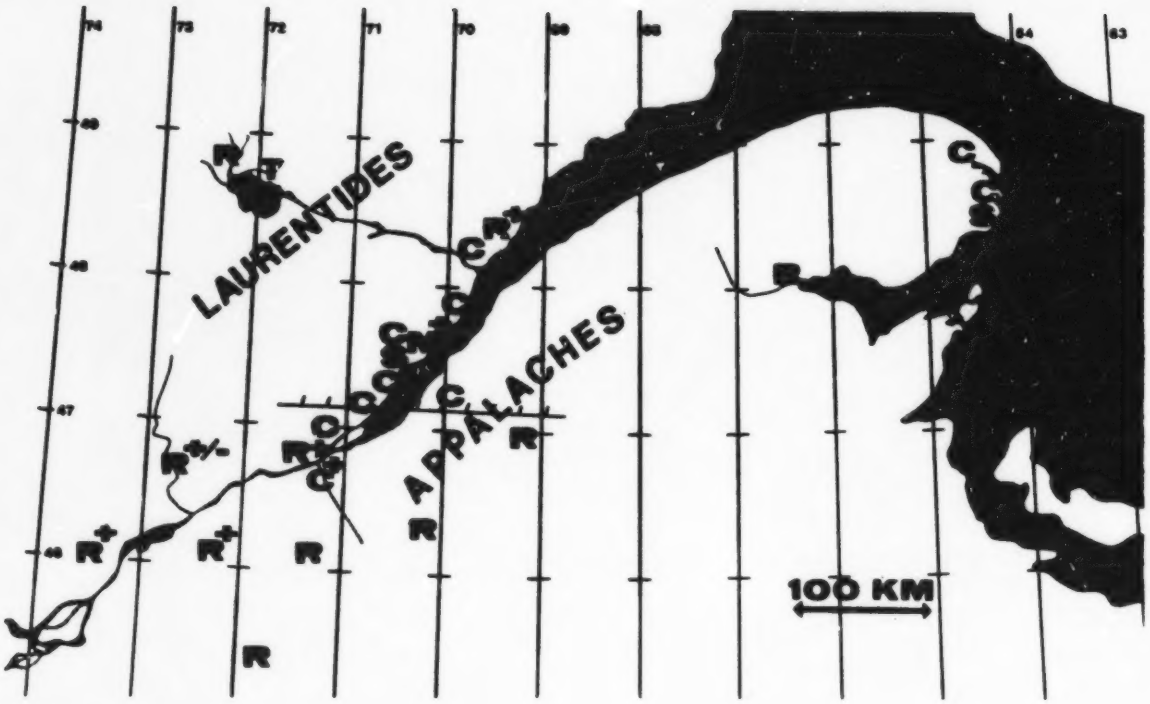
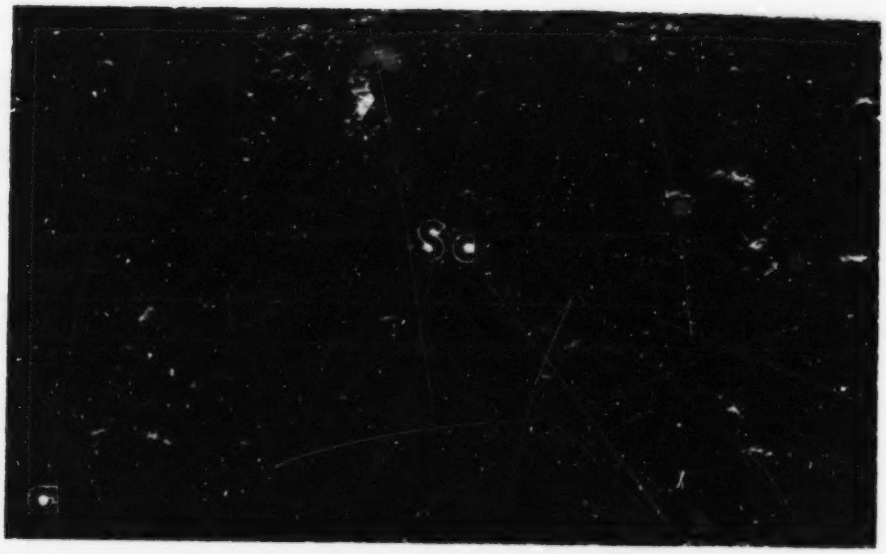


FIGURE 1. Map of southern Quebec showing the main water courses (dark), the St-Lawrence and Saguenay flood plains (white), and the above 500' highlands (light gray). "R" stands for provenances of *Alnus rugosa*, "C" for provenances of *A. crispa*, "M" for provenances of *Myrica gale*, "S" for the provenances of *Shepherdia canadensis*, and "+" for isolates obtained from soil inoculated to *A. crispa* seedlings. The "+" placed after the letter means that the isolates were of the Sp^+ type, "+/−" that both types of isolates were obtained and no sign placed there means that the isolates were of the Sp^- type. The hatched line represents the approximate southern limit of widespread occurrence of *A. crispa* on both sides of the river on the 47° parallel.



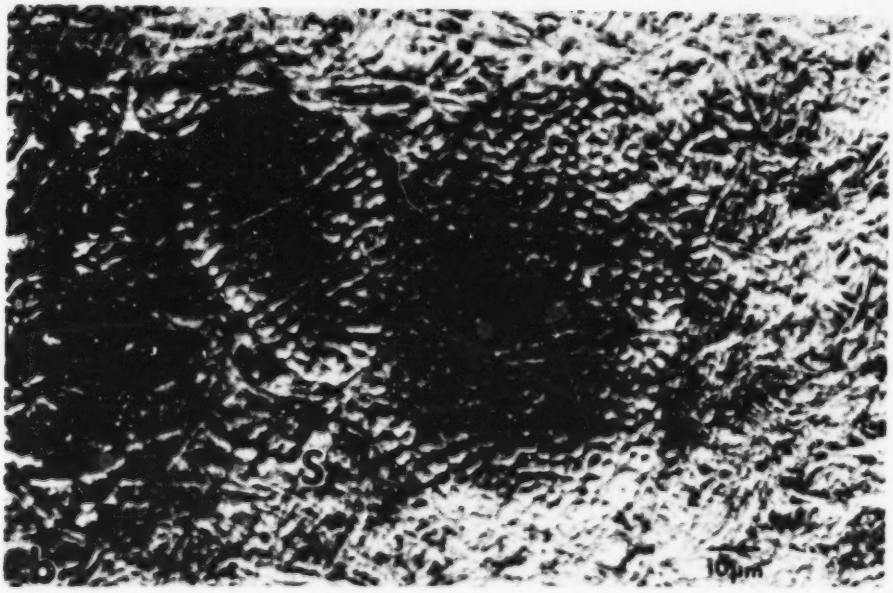


FIGURE 2a. Picture of the isolate ACN14a, typical of most Sp^- isolates, grown in Qmod B medium for 4 weeks. Interference contrast optics (bar = 50 µm). The spherical aggregates are sporangia clusters (Sc) against a backdrop of hyphae. FIGURE 2b. Sporangia (S) containing spherical, non-motile spores and hyphae of isolate ACN14a (bar = 10 µm).

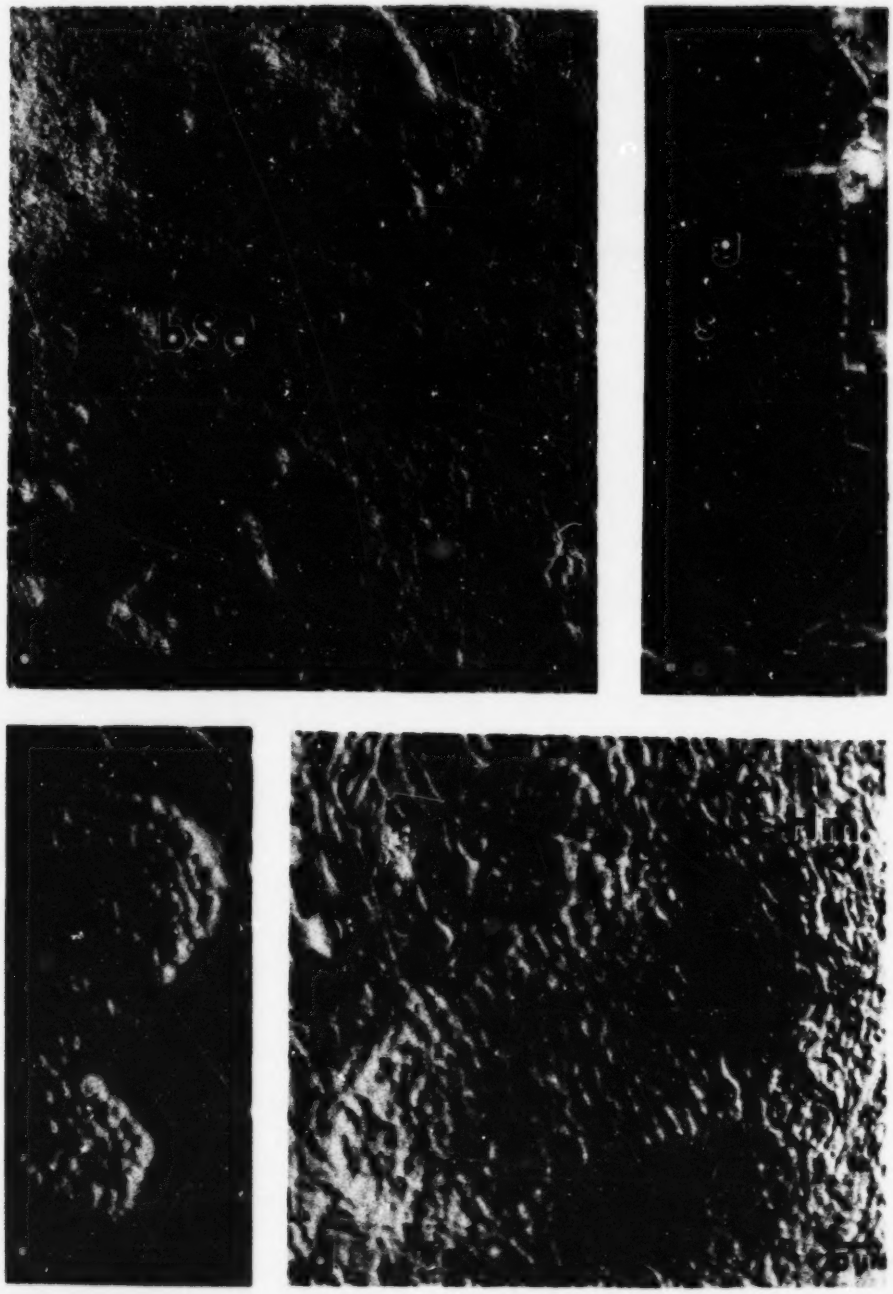


FIGURE 3a. Picture of colony of isolate MGP 10i, typical of Sp^+ isolates, grown in Qmod B-FBS medium for 3 weeks (bar = 50 μm). The darker shapes are sporangia clusters (bSc) imbedded in a thick hyphal matrix. FIGURE 3b. Sporangia growing outside the hyphal matrix, showing differential development of spores from bottom to top (bar = 10 μm). FIGURE 3c. Hyphae showing their septations(s) after treatment with acetic acid (30%) to dissolve Qmod B $CaCO_3$ crystals. The lumps in the hyphal shrunken cytoplasm are probably lipid granules (g) (bar = 10 μm). FIGURE 3d. Sporangia (s) imbedded in the dense hyphal matrix (μm); a rare vesicle (v) is present (bar = 10 μm).

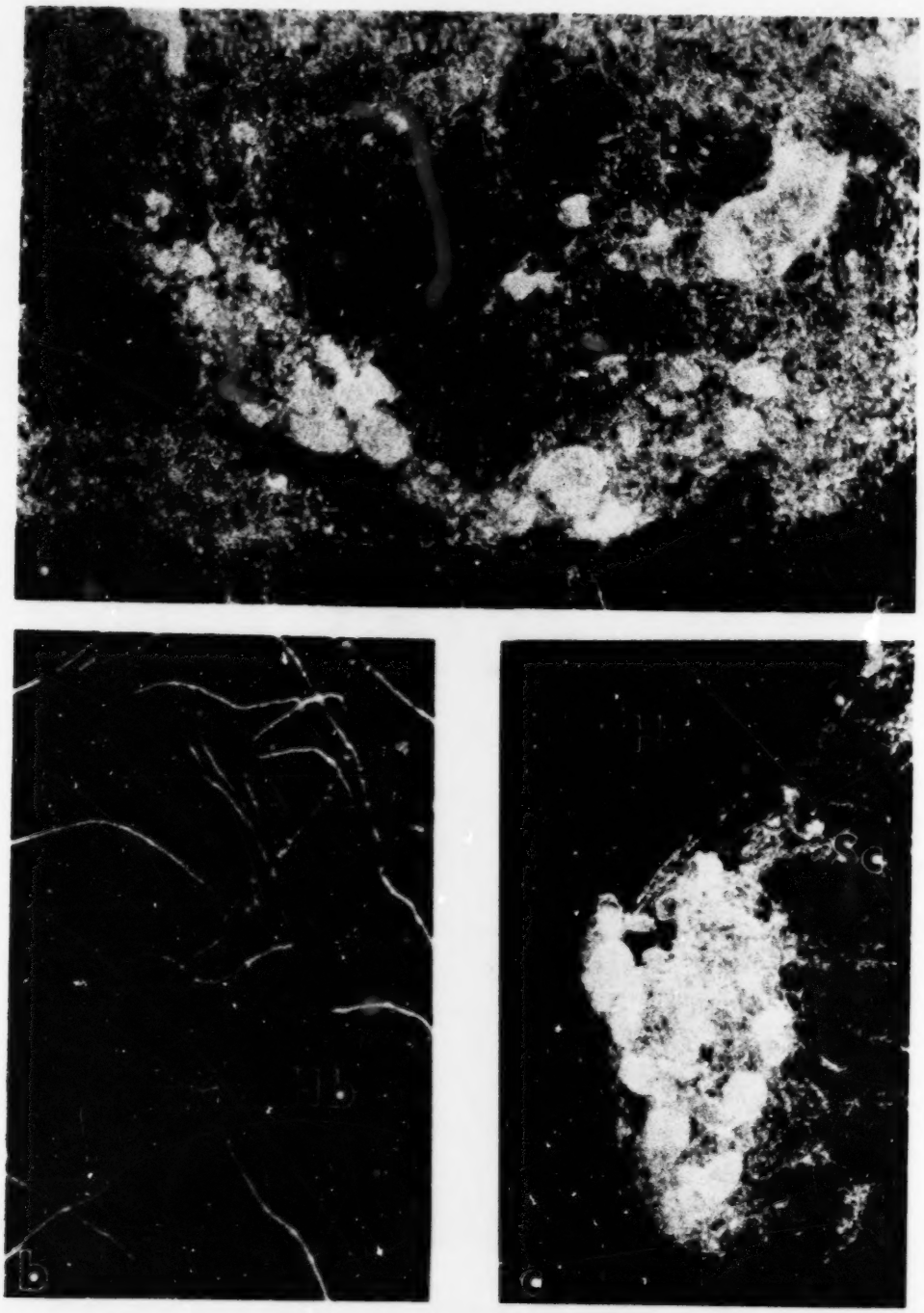


FIGURE 4a. Big and numerous sporangia (bS) of *Frankia* isolate ARgP5AG grown in Qmod B for three weeks (bar = 50 μm). FIGURE 4b. Hyphae showing branching (Hb) and lipid granules (bar = 10 μm). FIGURE 4c. Hyphae (H) and sporangia clusters (Sc) of a colony of ARgP5AG. Note size and density of sporangia (bar = 50 μm).

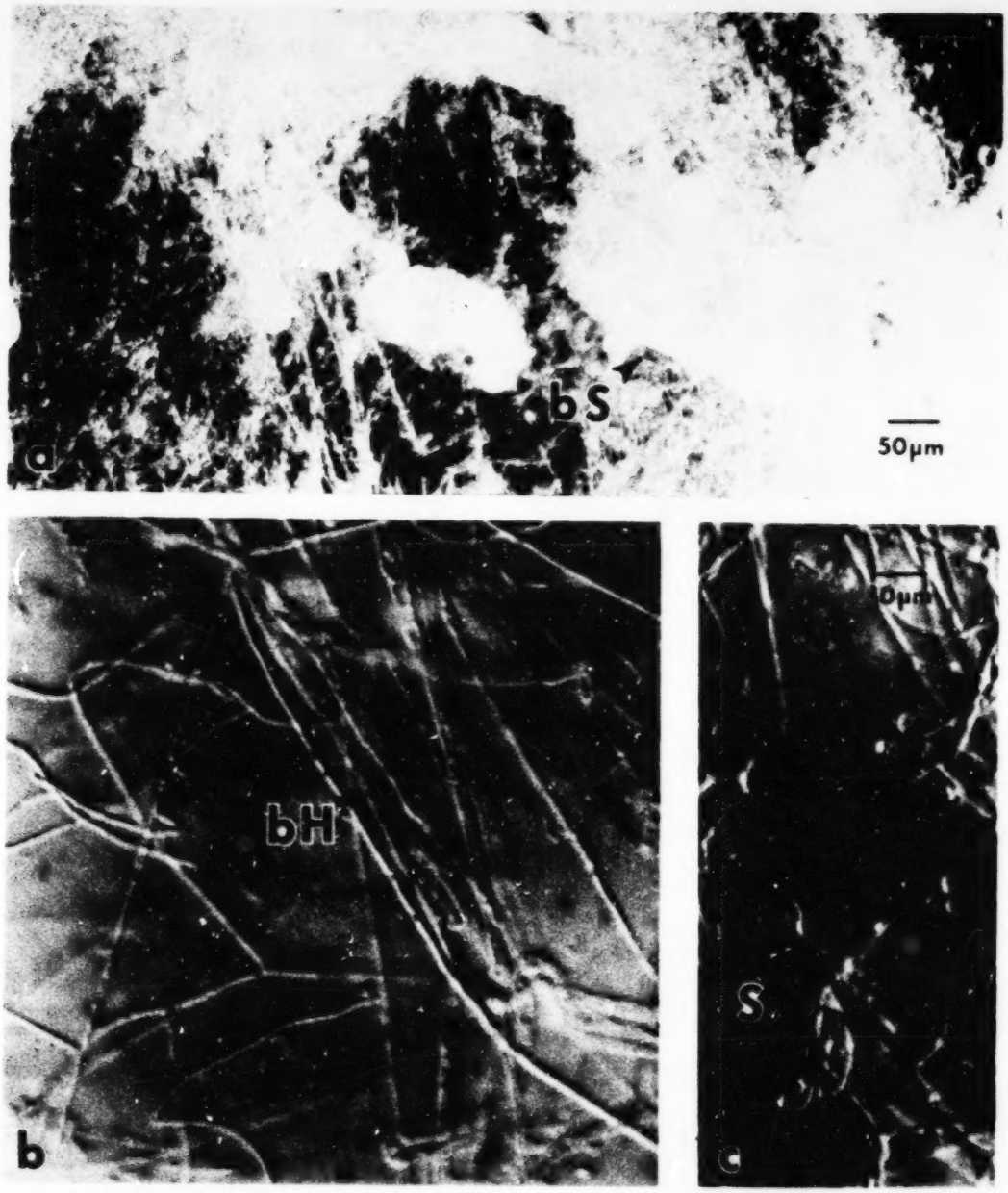


FIGURE 5a. Big sporangia of *Frankia* isolate ARgP5AGAC, which was reisolated from *A. crispa* inoculated with ARgP5AG, grown in Qmod B for three weeks. Note similarity between this figure and figure 4a (bar = 50 µm). FIGURE 5b. Big hyphae (bH) which are young developing intercalary sporangia (bar = 10 µm). FIGURE 5c. Intercalary sporangia (S) at beginning of maturation (bar = 10 µm).

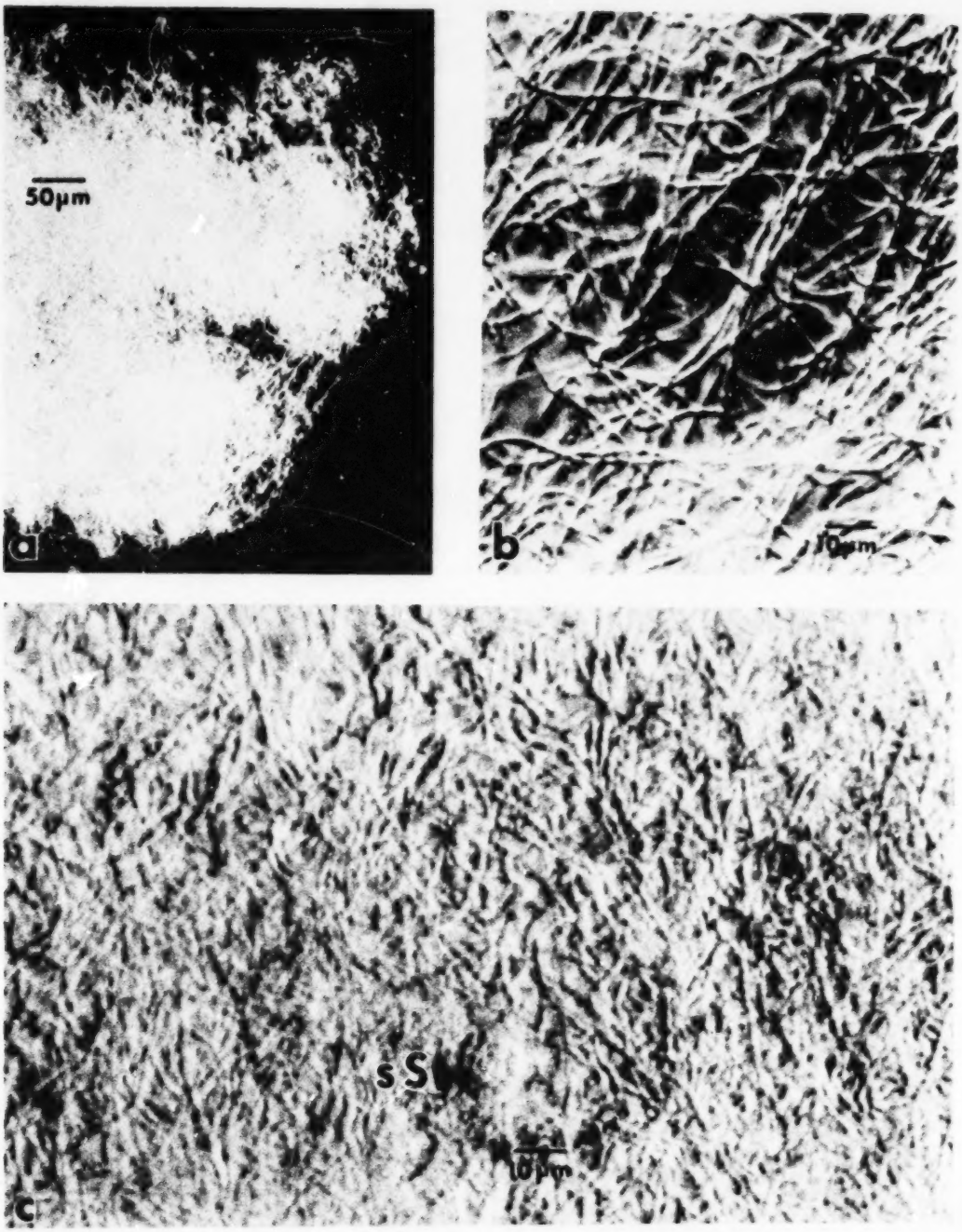


FIGURE 6a. Colony of *Frankia* isolate ACN8a grown in Qmod B medium for 3 weeks (bar = 50 µm). FIGURE 6b. Higher magnification of 6a. Typical field without sporangia (bar = 10 µm). FIGURE 6c. Rare small sporangium (sS, the only one found in that colony (bar = 10 µm)).

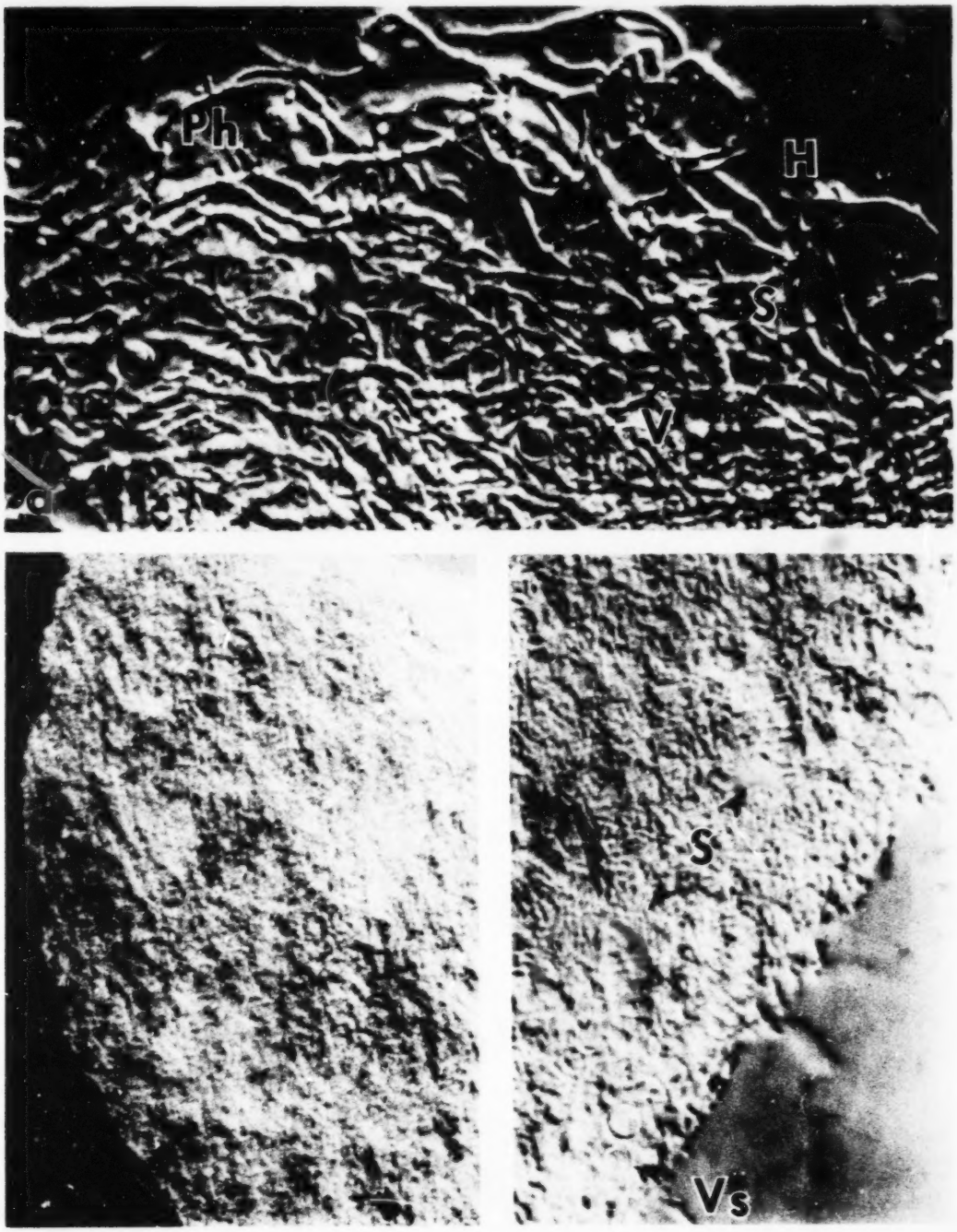


FIGURE 7a. Isolate SCN10a grown in Cmod B medium for three months. Note the number of vesicles. Interference contrast optics (bar = 10 μm); "S" stands for sporangium, "pH" for vesicle parental hypha, "H" for branched hypha and "V" for vesicle. FIGURE 7b. Note thickness and compactness of SCN10a colony, which is due to a matrix seemingly of a polysaccharide nature (bar = 50 μm). FIGURE 7c. Small sporangia (S) and vesicles (V) imbedded in the matrix (bar = 10 μm).

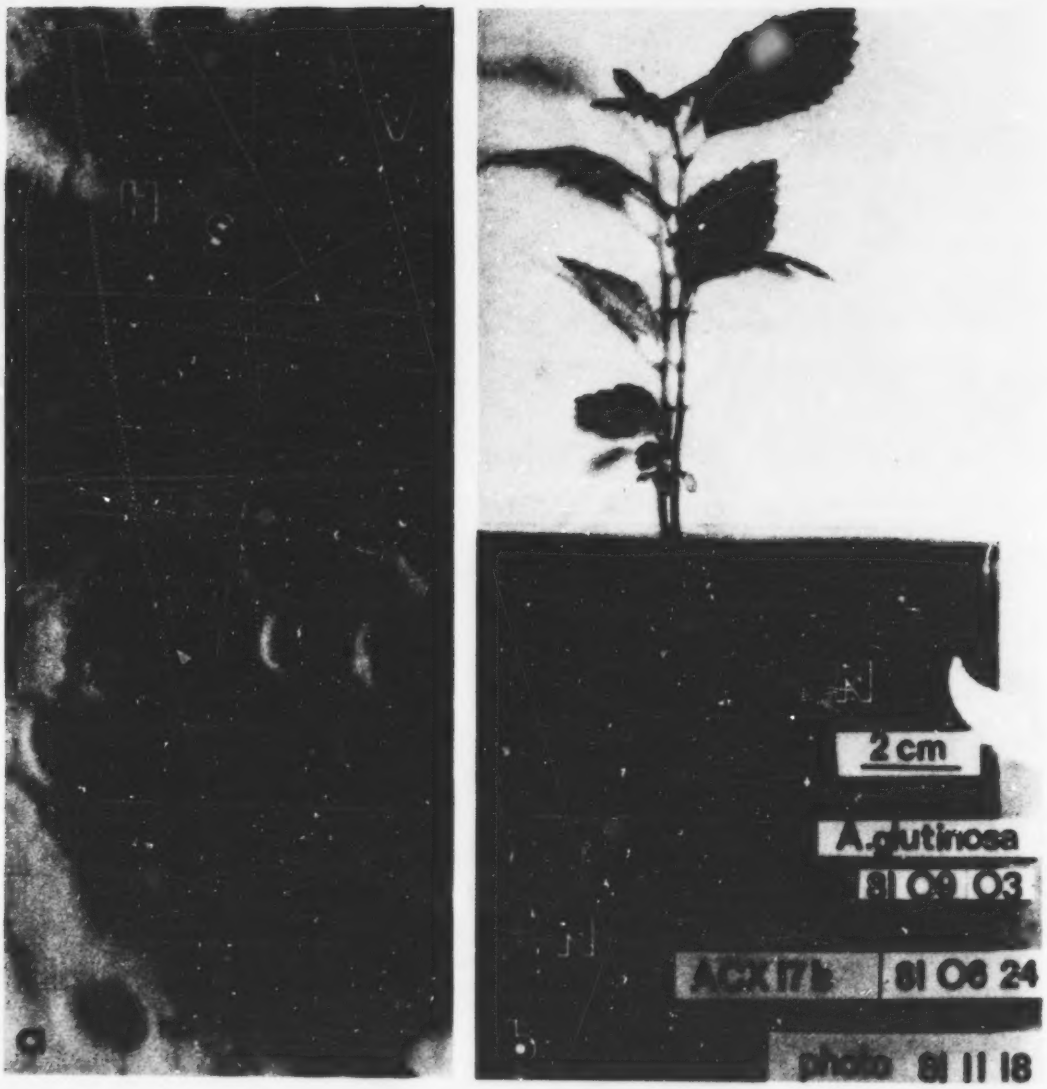


FIGURE 8a. Section of Sp+ type nodule showing inter-and intracellular sporangia. FIGURE 8b. Growth pouch used for testing infectivity of *Frankia* isolates.

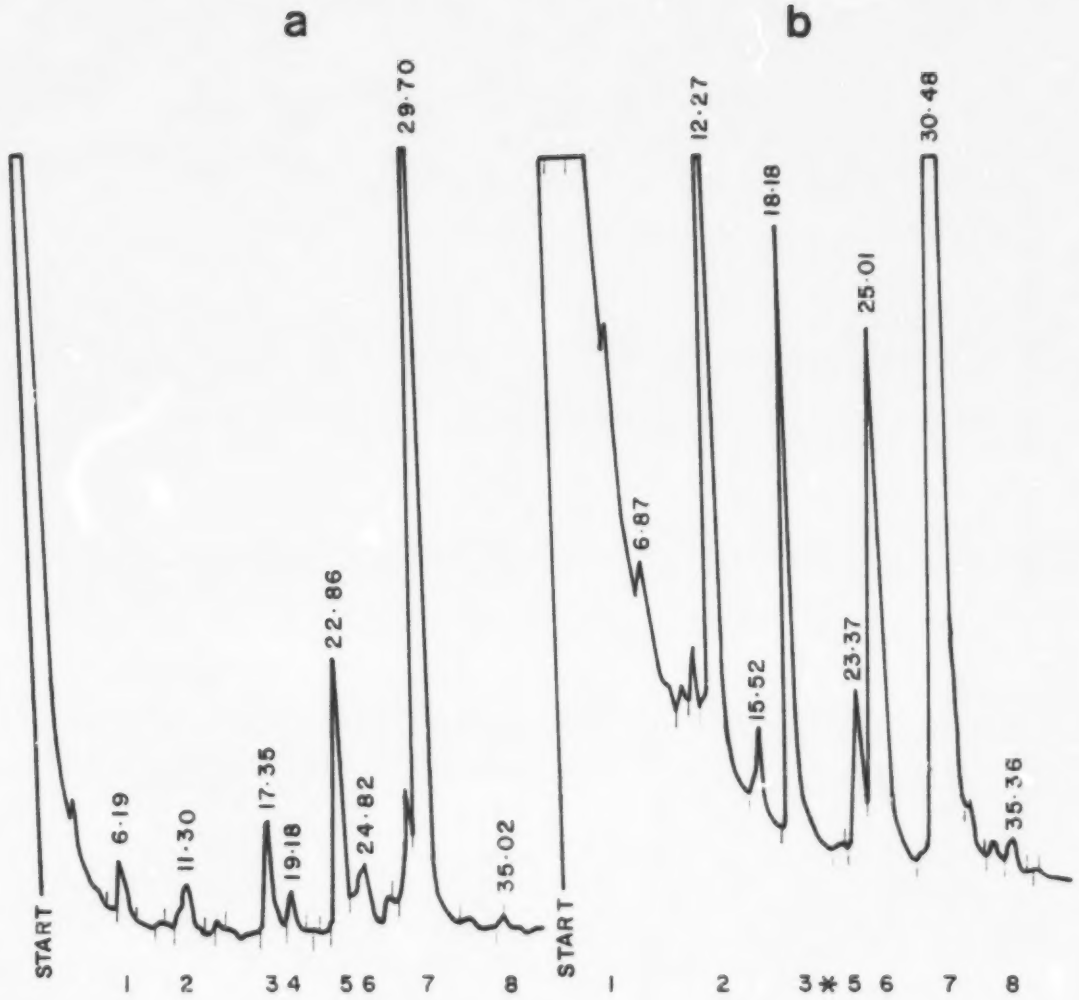


FIGURE 9. Chromatograms of alditol acetate derivatives of whole cell hydrolysates of *Frankia* strain TN15a^{AC}(A) and of *Actinoplanes philippinensis* (B) obtained according to the method given in the text (section 3.2.3). The peaks are, in order, (1) rhamnose, (2) ribose, (3) xylose, (4) 2-O-methyl-D-mannose, (5) mannose, (6) galactose, (7) glucose, and (8) inositol. The asterisk indicates the retention time of 2-O-methyl-D-mannose absent from the *Actinoplanes* chromatogram.

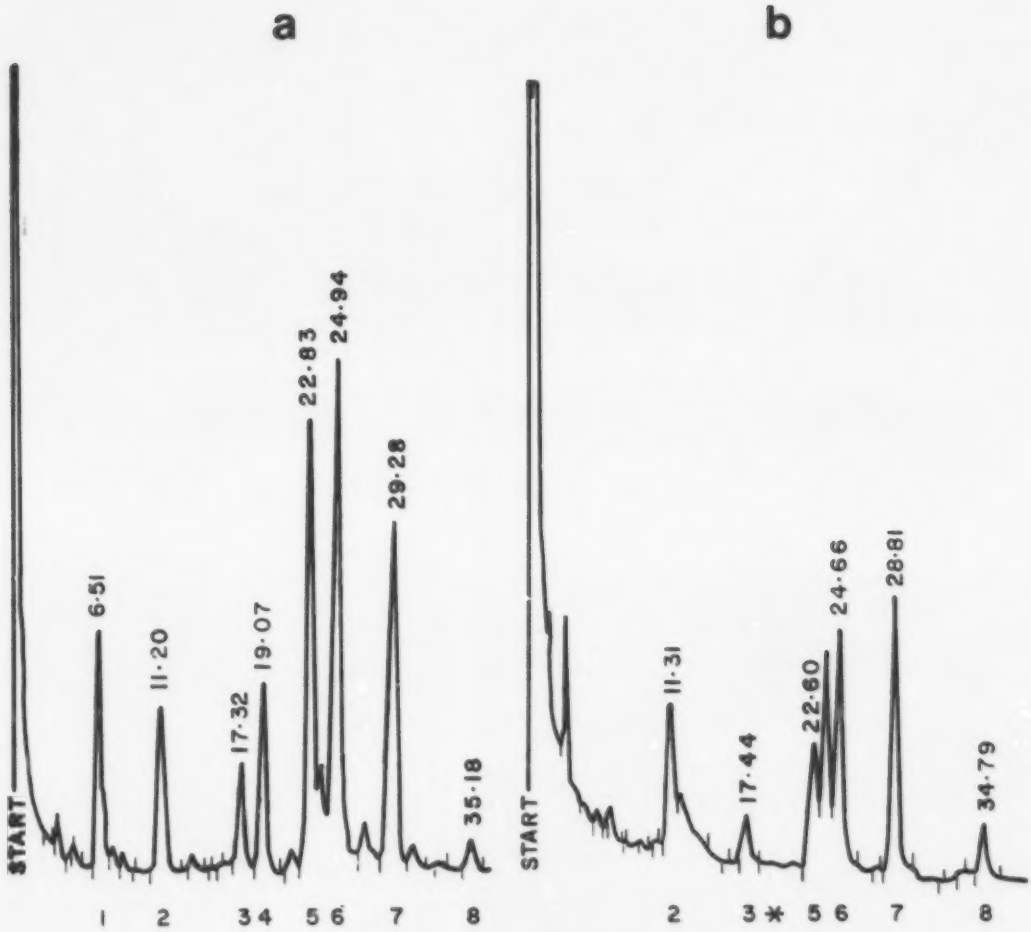


FIGURE 10. Chromatograms of alditol acetate derivatives of the "wall-filter fraction" (A) and of the "0.22 m filter fraction" of *Frankia* strain (B) ARgP5AG. The asterisk indicates where the 2-0-methyl-D-mannose peak should have been in the filtrate fraction.

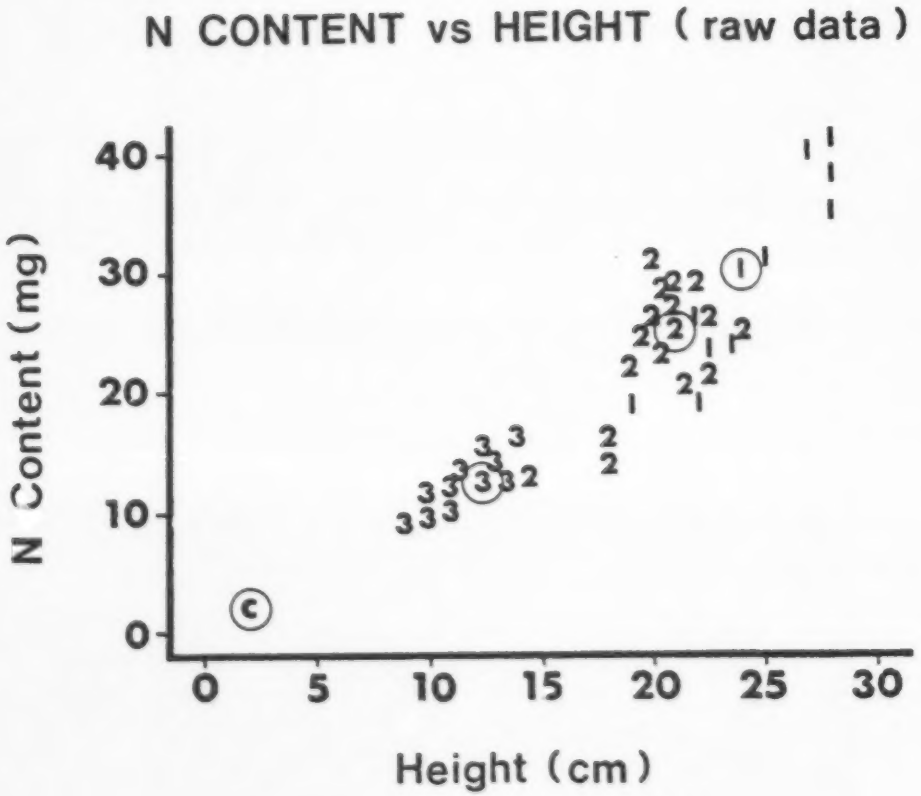


FIGURE 11. Graph of the N content vs. height, illustrating the variation between the two parameters. The plotted numbers refer to the three successive tests (# 1, # 2, and # 3) and are averages of each isolate. The circled "1", "2", and "3" are the overall averages of each test to show the difference caused by different growth conditions (differences in temperature and difference in inoculation delay after sowing). "C" is the average of all controls.

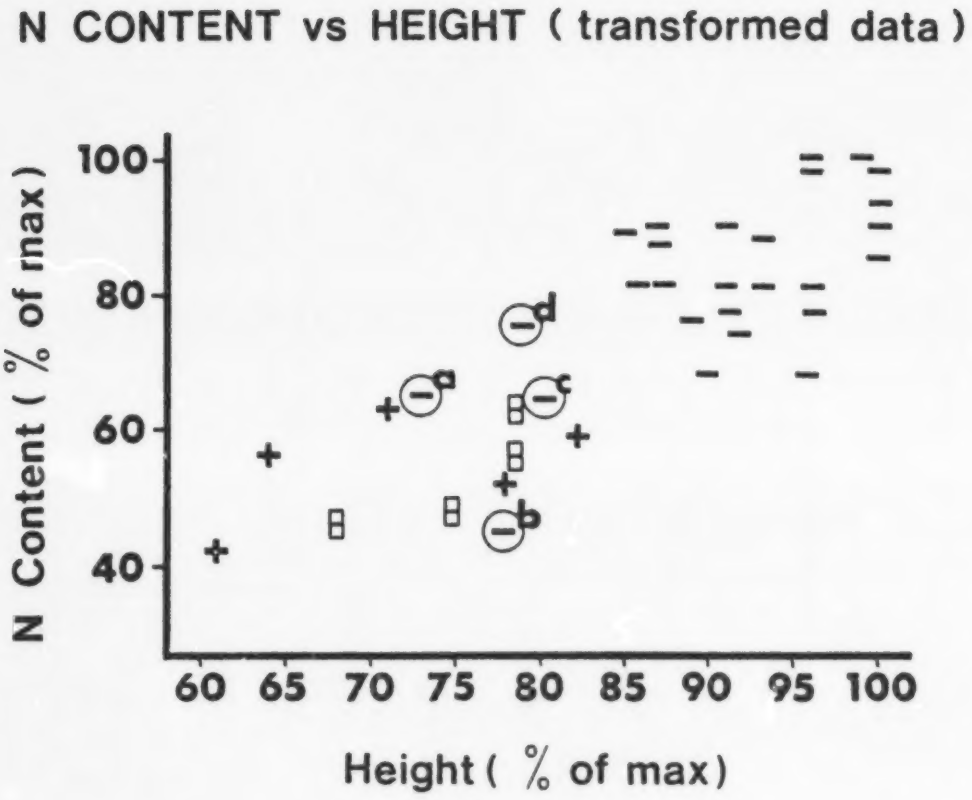


FIGURE 12. Graph of height vs. N content of shoots, both expressed as percent of the highest in each efficiency test. "+" refers to the Sp+ type isolates, "−" to the Sp− type isolates, "8" to the ACN8 isolates and the "0" are Sp− type isolates falling within the Sp+ cluster (1, ARgN18d; 2, ACN11b; 3, ARgN14a and 4, TN15$\frac{AC}{d}$).

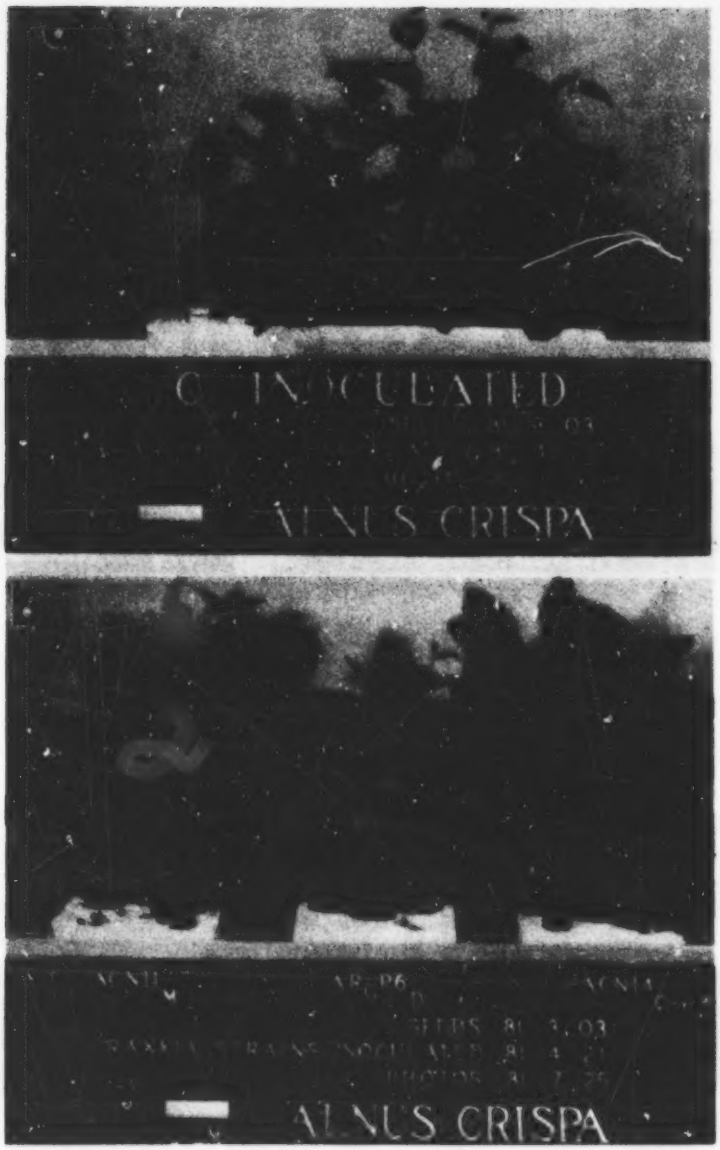


FIGURE 13a. *Alnus crispa* seedlings inoculated with ARgP6d *Frankia* isolate and controls (c) at left which were not inoculated; all plants were grown in Turface-filled styroblocks # 8. FIGURE 13b. *A. crispa* inoculated with ACN11m, ARgP6d and ACN14c. Note difference in height and vigour between the centre styroblock seedlings inoculated with a Sp+ type isolate and those on the sides inoculated with more efficient Sp− type isolates.

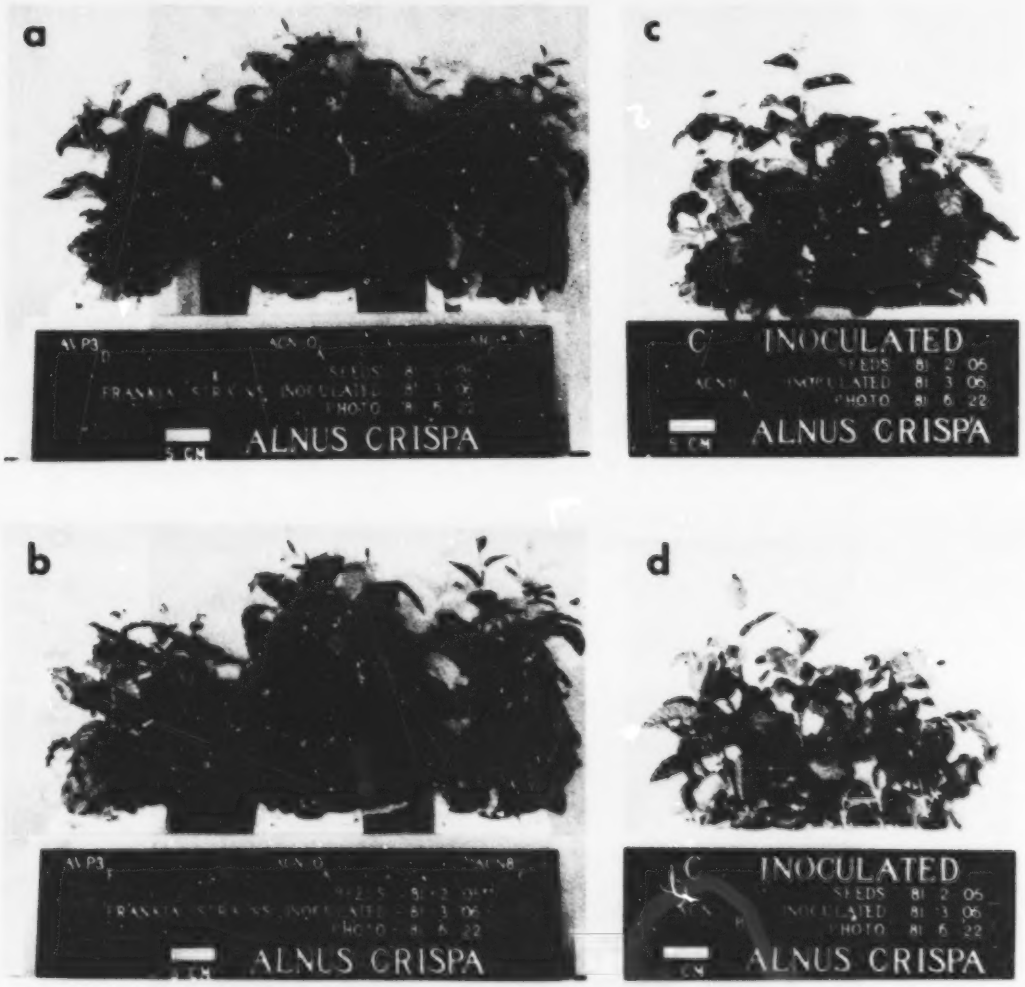


FIGURE 14a. *A. crispa* seedlings inoculated with AVP3d, ACN10a and ARgP5AG isolates. ACN10a, a Sp⁻ type isolate was the most efficient isolate tested in thest # 1, ARgP5AG, a Sp⁺ was intermediate and AVP3d, another Sp⁺ was lowest. **FIGURE 14b.** *A. crispa* seedlings inoculated with AVP3f, ACN10a and ACN8c isolates. AVP3f and ACN8c had comparable effectivities even though AVP3f was a Sp⁺ and ACN8c a Sp⁻. **FIGURES 14c and 14d.** *A. crispa* seedlings inoculated with ACN11a (14c) and ACN11b (14d). Note the difference in height and foliage density between the two, which are both from the same provenance.

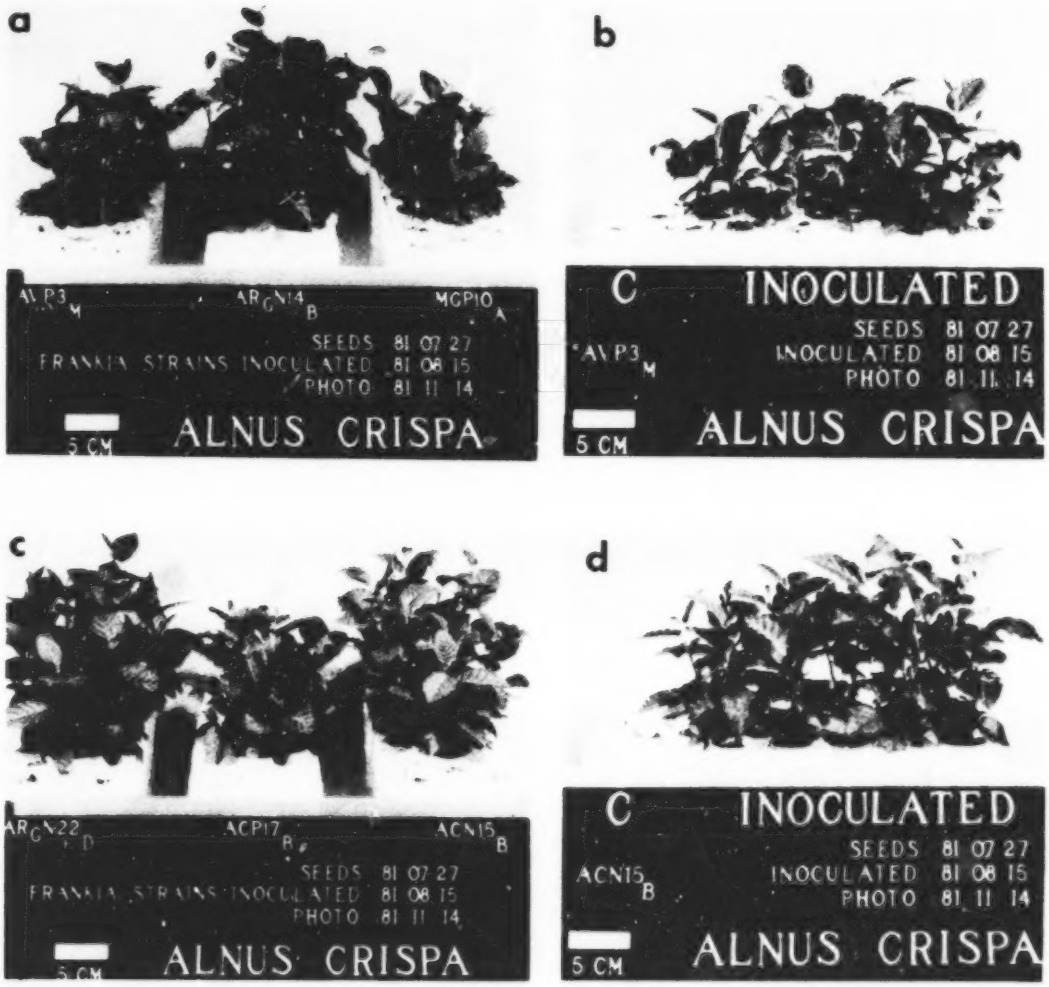


FIGURE 15a. *A. crispa* seedlings inoculated with AVP3m, ARgN14b and MGP10a isolates. MGP10a was the least effective of test # 3, AVP3m was second lowest and ARgN14b was one of the most effective isolates. FIGURE 15b. Side view of AVP3m inoculated *A. crispa* seedlings. Note border effect, but also the low amplitude of variation and the stunted N-starved controls (c). FIGURE 15c. *A. crispa* inoculated with ARgN22d, ACP17b and TN15$\frac{AC}{b}$(ACN15b in the picture, the acronym was changed later). Effectivity was not related to host origin spore type. FIGURE 15d.] Side view of TN15$\frac{AC}{b}$(ACN15b) inoculated seedlings.

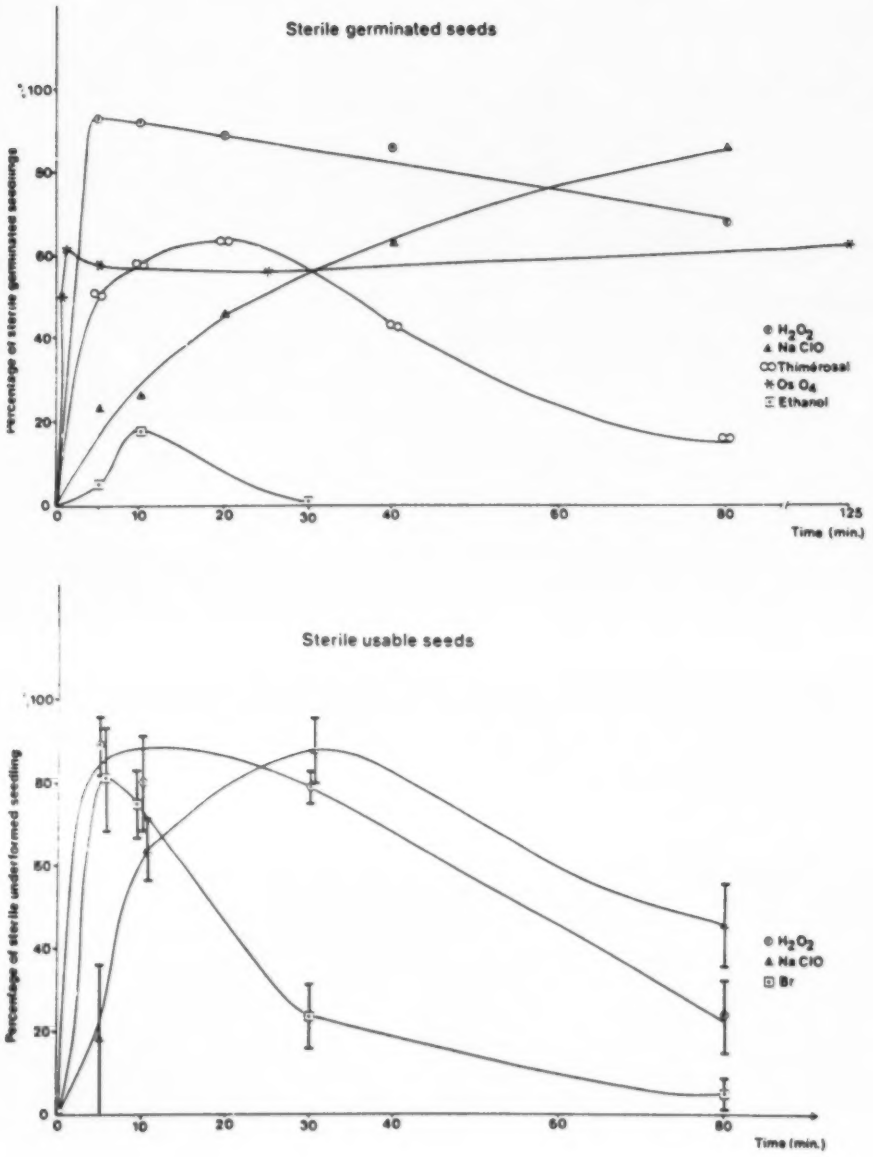


FIGURE 16a. The percent of axenic germinated *A. crispa* seedlings five days after various surface sterilization procedures. The germination rate of the control on quartz sand was 95%. FIGURE 16b. The percent of seeds giving rise to axenic and normal seedlings (mean ± SD), control germination on quartz sand was 95%. The difference in the shape of the H_2O_2 curve with the previous figure is due to the fact after long immersion periods, an increase in the number of deformed seedlings was observed. The difference in NaClO curve optima is caused by the mode of action of hypochlorite that digests its way into the teguments and produces erratic results.